INVESTIGATING MAIZE INBRED LINE RESPONSES FOLLOWING INFECTION BY THE MYCOTOXIGENIC FUNGUS *FUSARIUM VERTICILLIOIDES*

by

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DECLARATION

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SUMMARY

Fusarium verticillioides is an important fungal pathogen of maize and is associated with the crop wherever it is produced. It is the most common fungal contaminant of South African maize grain and causes Fusarium ear rot (FER). The presence of *F. verticillioides* can reduce grain yield and quality by the visible moulding and/or discolouration of the kernels which reduces its grading at silos. The fungus may also contaminate grain without visible symptoms and produce harmful secondary metabolites known as fumonisins. Fumonisins have been associated with a number of noxious effects on humans and animals. Their widespread occurrence in maize and maize-based products has led to numerous countries imposing maximum allowable limits of fumonisins in food and feed. Cultural practises, mainly focussed on optimal plant production as a disease management strategy, have been shown to reduce fumonisin contamination. However, host-plant resistance is considered the most feasible, economical and environmentally sound approach to manage *F. verticillioides* and its fumonisins.

In this study the response of elite breeding lines to infection and fumonisin deposition by *F. verticillioides* was evaluated in a multi-environment trial over 2 years. These lines were evaluated with inbred lines previously characterised for their response to FER and fumonisin accumulation. The stability of the inbred line response across locations was also determined in order to identify lines with broad or specific adaptability for disease resistance. Inbred lines CML 390, RO 424W, US 2540W and VO 617y-2 consistently exhibited low FER severity (≤5%), fungal target DNA (≤0.1 ng μL^{-1}) and fumonisin levels (≤5 mg kg^{-1}) at most test locations. Line RO 424W was the most stable in its response to disease and fumonisin accumulation across environments. The elite inbred lines were highly susceptible to *F. verticillioides* and fumonisin accumulation with only CB-222 and CB-248 having intermediate resistance. These inbred lines may serve as sources of resistance in breeding programmes but can also be used in genomic and proteomic studies to better understand the genetic basis of resistance to *F. verticillioides* and its fumonisins.

The response of Kenyan inbred lines, previously characterised for resistance to *Aspergillus flavus* and aflatoxins, to *F. verticillioides* and fumonisin contamination was evaluated in South Africa and Kenya. Common resistance mechanisms to these pathogens have been reported. The AER/aflatoxin-resistant lines were compared to South African lines previously characterised as resistant to *F. verticillioides* and fumonisin accumulation. Kenyan inbred lines CML 495, CML 264 and CKL05015 were most resistant to FER, *F. verticillioides* colonisation and fumonisin accumulation across localities. The inbred line CML 495 was also the most stable in its resistance response to *F. verticillioides* infection and fumonisin

deposition, making it an attractive source of resistance for inclusion into a South African breeding programme. This study, therefore, provided further evidence that AER/aflatoxin-resistant lines appear to be a significant source of resistance to *F. verticillioides* and fumonisins.

Genetic variability for resistance to *F. verticillioides* and fumonisin accumulation was generated by gamma irradiation of seven elite maize lines. Following mass irradiation, the lines were field planted and self-pollinated for four consecutive seasons (M_0-M_4 generations). The response of the M_1 to M_4 populations to *F. verticillioides* was evaluated visually for FER severity annually, and ears expressing less than 10% FER were advanced to the next breeding cycle. The M_4 selections were also evaluated for *F. verticillioides* colonisation and fumonisin content. A number of selections derived from each inbred line were more resistant to FER, *F. verticillioides* colonisation and fumonisin accumulation when compared to their non-irradiated controls. However, no improved selections were obtained from inbred I-35.

The transcriptional changes in maize induced upon infection by *F. verticillioides* were evaluated by next-generation RNA sequencing and monitored up to 7 days after inoculation (dai). Although an initial induction of defence-related transcripts associated with pathogen recognition, signalling molecules, pathogenesis-related genes, cell wall restructuring and secondary hormone-based signalling genes was observed 24 hours post inoculation (hpi), these were down-regulated 48 hpi. Plant responses did not prevent *F. verticillioides* from colonising maize kernels, as the target DNA of the pathogen continued to increase. At 72 hpi, genes involved in pathogenesis, G-coupled receptor signalling and response to oxidative stress were induced and may have resulted in the reduction of fungal contamination 7 dai. The transcriptional changes in maize suggest a delayed plant response to *F. verticillioides* infection and imply a pathogen-associated molecular pattern response characterised by a basal immunity. Several genes including PRRs, signalling molecules (protein kinases, calcium-dependant molecules, GTP-signalling and redox-associated molecules), PR protein-coding genes and those involved in secondary hormone signalling (auxins) that influence maize response to *F. verticillioides* warrant further investigation by genomic and proteomic approaches.

OPSOMMING

Fusarium verticillioides is 'n belangrike patogeniese swam van mielies en word ge-assosïeer met die gewas in alle produksie areas wêreldwyd. Dit is die mees algemene swamsiekte van Suid-Afrikaanse mielie graan en die swam kan Fusarium kop vrot (FKV) veroorsaak. Die teenwoordigheid van *F. verticillioides* kan graanopbrengs en gehalte verlaag deur die sigbare besmetting en/of verkleuring van die pitte wat die gradering by silo's verlaag. Die swam kan ook graan besmet in die afwesigheid van sigbare simptome. Verder, kan dit skadelike sekondêre metaboliete, bekend as fumonisiens, produseer. Fumonisiens word ge-assosïeer met verskeie gesondheidsrisikos van mense en diere. Die wydverspreide voorkoms van fumonisiens in mielies en mielie produkte het gelei tot die instelling van maksimum toelaatbare vlakke van fumonisiens in voedsel en voer in talle lande. Bewerkings praktyke, veral gefokus op optimale plantproduksie as 'n siektebestuur strategie, het getoon dat dit fumonisiens besoedeling kan verminder. Dit is egter gasheerplant weerstand wat beskou word as die mees haalbare, ekonomiese en omgewingsvriendelike benadering tot *F. verticillioides* en sy fumonisiens.

In hierdie studie is die reaksie van mielie teellyne teenoor infeksie en fumonisien neerlaag deur *F. verticillioides* in 'n multi-omgewing proef oor 2 jaar geëvalueer. Hierdie mielie lyne was vergelyk met lyne wat voorheen gekaraktiseerd is vir hul reaksie op FKV en fumonisiens. Die reaksie stabiliteit van die teellyn oor die verskillende lokaliteite is ook bepaal om lyne met 'n breë of spesifieke aanpasbaarheid vir siekte weerstand te identifiseer. Die lyne CML 390, RO 424W, VSA 2540W en VO 617y-2, voorheen bepaal as weerstandbiedend, het konstant lae FKV (≤5%), swam teiken DNA (≤0.1 ng µL-1) en fumonisien vlakke (≤5 dpm) by die meeste omgewings getoon. Lyn RO 424W was die mees stabiel in sy reaksie op die siekte en fumonisiens opeenhoping oor alle omgewings. Die ongekaraktiseerde teellyne was hoogs vatbaar vir *F. verticillioides* en fumonisiens opeenhoping met slegs CB-222 en CB-248 wat intermediêre weerstand getoon het. Die weerstandbiedende lyne wat in hierdie studie ge-identifiseer was kan dien as bronne van weerstand in teelprogramme, maar kan ook gebruik word in genomiese en proteomiese studies om die genetiese basis van weerstand teenoor *F. verticillioides* en sy fumonisins beter te verstaan.

Die reaksie van Keniaanse mielie teellyne, voorheen gekenmerk vir weerstand teen *Aspergillus flavus* (wat Aspergillus kop vrot [AKV] veroorsaak) en aflatoksiene, om *F. verticillioides* en fumonisiens besmetting te weerstaan is in Suid-Afrika en Kenia geëvalueer. Algemene weerstand meganismes tot hierdie patogene is voorheen aangemeld. Hierdie AKV/aflatoksien-weerstandbiedende lyne was met die FKV/fumonisien- weerstandbiedende

Suid-Afrikaanse lyne vergelyk. Keniaanse teellyne CML 495, CML 264 en CKL05015 was die mees weerstandbiedend teen FKV, *F. verticillioides* kolonisasie en fumonisiens opeenhoping oor lokaliteite. Die lyn CML 495 was ook die mees stabiel in sy weerstandreaksie op *F. verticillioides* infeksie en fumonisiens en maak dit 'n aantreklike bron van weerstand vir insluiting in 'n Suid-Afrikaanse teelprogram. Hierdie studie lewer dus verdere bewyse dat AKV/aflatoksien-weerstandige lyne 'n belangrike bron van weerstand teen *F. verticillioides* en fumonisins kan wees.

Genetiese variasie van sewe mielie teellyne, met die oog om weerstand teen *F. verticillioides* en fumonisins opeenhoping te genereer, is aan gamma bestraling blootgestel. Die massa bestraalde lyne was in die veld geplant en self-bestuif vir vier agtereenvolgende seisoene (M_0-M_4 geslagte). Die reaksie van die M_1 tot M_4 generasies op *F. verticillioides* infeksie is visueel beoordeel vir FKV simptome en mielie koppe met minder as 10% FKV is tot die volgende teelsiklus oorgedra. Geselekteerde M_4 koppe is ook vir kolonisasie en fumonisins inhoud geëvalueer. 'n Aantal geselekteerde M_4 mieliekoppe, afgelei van elke teellyn, was meer bestand teen FKV, *F. verticillioides* kolonisasie en fumonisiens opeenhoping in vergelyking met hul onbestraalde oorspronklike ouer teellyn. Die bestraalde nageslag van lyn I-35 was nie meer siektebestand nie.

Die transkripsionele veranderinge in mielies, as gevolg van infeksie deur *F. verticillioides*, is geëvalueer deur die volgende generasie RNA basispaarvolgorde en gemonitor vanaf nul tot 7 dae na inokulasie (dni). Aanvanklik is verdedigings gene wat verband hou met patogeen erkenning, sein molekules, patogenese verwante gene, herstrukturering van die selwand en sekondêre hormoon gebaseer sein gene 24 uur na inokulasie (uni), geïnduseer. Hierdie gene is egter 48 hni af-gereguleer. Hierdie plant reaksies het nie verhoed dat *F. verticillioides* mieliepitte koloniseer nie, soos aangedui deur die verhoging van die patogeen teiken DNA. Op 72 hni, was gene betrokke in patogenese, G-gekoppelde reseptorsein en reaksie op oksidatiewe stres geïnduseer en mag dit gelei het tot die vermindering van die swam besmetting op 7 dni. Die transkripsionele veranderinge in mieliepitte dui op 'n vertraagde reaksie van die plant tot infeksie deur *F. verticillioides* en impliseer 'n patogeen geassosieerde molekulêre patroon (PAMP) reaksie gekenmerk deur 'n basale immuniteit. Verskeie gene, insluitend herkennings molekules, sein molekules (proteïenkinases, kalsium-afhanklike molekules, GTP-sein en redoks ge-assosïeerde molekules), PR-proteïen koderende gene en die gene wat betrokke is in sekondêre hormoon sein (ouksiene) wat mielies se reaksie op *F. verticillioides* beinvloed, benodig verder ondersoek deur genomiese en proteomiese benaderings.

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CHAPTER 1

Resistance in maize to mycotoxigenic ear rot pathogens in Africa, with special reference to *Fusarium verticillioides*

INTRODUCTION

Maize (*Zea mays* L.) constitutes a major food crop grown worldwide and is amongst the most important staple foods produced in Africa (Byerlee and Eicher, 1997). Between 2008 and 2010 the African continent contributed approximately 7.5% to global maize production (FAOSTAT, 2014). The production and quality of maize in Africa, however, is influenced by a number of agricultural constraints, including a lack of irrigation water, and limited access to quality seed, fertilisers and insecticides (Smale *et al*., 2011). The widespread contamination of maize with mycotoxigenic fungi further poses a food safety concern. The maize-based diets and high daily consumption of grain often result in in the excessive intake of mycotoxins, even when the grain is only moderately contaminated (Shephard, 2004). Under severe contamination levels, however, outbreaks of mycotoxicosis can result in human fatalities (Lewis *et al*., 2005).

The contamination of maize grain produced in Africa with mycotoxins is due to their association with major mycotoxigenic fungi, including *Fusarium verticillioides* (Sacc.) Nirenberg (syn. *F. moniliforme* Sheldon), *Aspergillus flavus* (Link:Fr) and *Fusarium graminearum* Schwabe. Of these, *F. verticillioides* is most commonly associated with maize in southern and western Africa (Fandohan *et al*., 2003, 2005a). The fungus causes Fusarium ear rot (FER), along with *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas and *F. proliferatum* (Matsushima) Nirenberg (Flett, *et al*., 1998; Rheeder *et al.*, 2002; Ncube *et al*., 2011; Boutigny *et al*., 2012). Maize grain from eastern and central Africa is more often contaminated with *A. flavus*, which causes Aspergillus ear rot (AER), while the occurrence of *F. graminearum*, the cause of Gibberella ear rot (GER), is mostly sporadic (Viger *et al*., 2001).

Strategies to reduce mycotoxin contamination of maize grain before harvest involve good agricultural practices followed by good manufacturing practices (CAC, 2003). An important component of managing mycotoxins before harvest is the planting of genotypes that are agronomically suited to the production region, and with adequate resistance to ear rot pathogens and their associated mycotoxins. The identification of such genotypes should, therefore, be a priority of maize breeding programmes in Africa. Resistant genotypes could also aid in the identification of resistance genes or mechanisms associated with resistance that could lead to a reduction of ear rot diseases and mycotoxins through conventional or genetic plant improvement strategies (Acquaah, 2007).

This review aims to highlight the importance of mycotoxigenic fungi in Africa by providing a comprehensive synthesis of the factors which contribute to their occurrence and management. Specific focus is placed on the *F. verticillioides*-maize interaction and recent developments to understand the genetic mechanisms of resistance in maize.

MAIZE PRODUCTION IN AFRICA

Maize is the third largest crop produced on the African continent after cassava and sugarcane (FAOSTAT, 2014). Countries comprising eastern Africa have produced an average of 27 million tonnes (MT) of maize between 2009 and 2014 (FAOSTAT, 2014). The major maize-producing countries in this region are Ethiopia (5.8 MT), Tanzania (4.6 MT) and Kenya (3.3 MT). South Africa is the main maize producer in the Southern African Development Community (SADAC) region, and has produced an average of 12.4 MT for the period 2009-2014. This is more than any other African country. Western Africa produced an average of 16.7 million tonnes between 2009 and 2014, of which Nigeria produced 8.6 MT, Mali 1.5 MT, Ghana 1.7 MT and Benin 1.1 MT. The northern African countries contributed an average of 7.4 MT (2009-2014), with Egypt (7.2 MT) being the main maize-producing country in this region. Central African countries produced an average of more than 4.5 MT of maize during 2009-2014, with Angola (1.1 MT) and Cameroon (1.6 MT) producing the most maize (FAOSTAT, 2014).

Maize in southern African is largely produced by intensive, commercial farming while maize in the rest of Africa is produced by resource-poor subsistence farmers (Ncube *et al*., 2011, Janse van Rensburg *et al*., 2015). Production is primarily to meet the demands of local markets but, in the event of a surplus, maize is exported to other countries on the continent. Few countries in Sub-Saharan Africa are competitive in global markets, largely because of high transport and logistics costs (Smale *et al*., 2011). In recent years, access to improved open-pollinated varieties has significantly increased maize yield in Africa. Maize yield in Africa has increased from 20 000 hg/ha to nearly 21 000 hg/ha (FAOSTAT, 2014). The adoption of improved varieties in eastern and southern Africa, excluding South Africa, was estimated at 44% in 2006-07, and at 60% in western and central Africa in 2005 (Smale *et al*., 2011). The costs of fertilizer and chemical control of insects and pests is, however, still unaffordable to subsistence farmers. Furthermore, poor soil fertility, water scarcity, drought and heat stress threaten productivity and increase susceptibility to pathogenic microorganisms (Oerke and Dehne, 2004; Barnabas *et al*., 2008, Marin *et al*., 2010). Greater productivity and improved infrastructure could lead to the expansion of regional markets and eventually provide the basis for competition in export markets (World Bank, 2009).

EAR ROT DISEASES OF MAIZE

Pathogens that cause ear rot diseases represent a key biotic constraint to the increased production of maize in Africa (Hefny *et al*., 2012). The ear rot pathogens *F. verticillioides* and *A. flavus*, which cause FER and AER respectively, are considered the most important. Their associated mycotoxins, fumonisins and aflatoxins, are estimated to be the most widespread in Africa's major dietary staples (Wagacha and Muthomi, 2008). Other important ear rot diseases of maize that occur on the continent include GER caused by *F. graminearum*, Diplodia ear rot caused by *Stenocarpella maydis* (Berk.) B. Sutton and Penicillium ear rot caused by *Penicillium oxalicum* Currie and Thom and other *Penicillium* spp. These fungi also contaminate maize grain with their respective mycotoxins.

Fusarium ear rot (FER)

FER is an important disease of maize in southern and western African countries. It is caused by *F. verticillioides*, a ubiquitously-occurring fungus, which infects maize wherever it is grown. FER symptoms range from cotton-like white/grey to light pink fungal growth on individuals or clusters of maize kernels (Fig. 1) that are randomly distributed across the maize ear (White, 1999). Fungal growth within kernels may appear as white or pink streaks across the top of infected kernels, known as "starburst" symptom (Koehler, 1942; Duncan and Howard, 2010). In addition to the characteristic pre-harvest moulding of maize ears, *F. verticillioides* also causes rotting of maize stalks and seedlings (Kommendahl and Windels, 1981), or infects maize plants without the development of any visible symptoms (Foley, 1962). It is not known why asymptomatic infections occur or what triggers rotting in infected tissue (Bacon and Hinton, 1996; Yates *et al.*, 1997).

Fusarium verticillioides can infect a maize plant at any developmental stage. Systemic infection takes place via contaminated seeds (Foley, 1962; Sumner, 1968; Yates *et al.*, 1999) or through plant roots grown in infested soils (Desjardins, 2003). Its systemic nature allows the fungus to be transmitted from the planted seed to the kernels of mature plants (Munkvold and Carlton, 1997; Munkvold *et al.*, 1997). Asexual spores of *F. verticillioides* produced on maize debris from a previous season also serve as primary inoculum for above-ground infections (Smith and White, 1988). These spores are dispersed by wind, rain-splashed, and/or vectored by insects to cause FER and stem infections of maize plants (Ooka and Kommendahl, 1977; Munkvold and Desjardins, 1997; Munkvold, 2003b). Ear infections most commonly take place through exposed silks (Jones *et al*., 1980; Headrick and Pataky, 1991; Reid *et al*., 1992; Munkvold *et al*., 1997). Duncan and Howard (2010) hypothesized that the passive movement of conidia along the surface of silks, perhaps via capillarity forces, as a possible mechanism for pathogen access to the stylar canal, which they showed to serve as an infection court. Insect and animal damage also

provides wounds for *F. verticillioides* to infect ears and stems of maize plants (Warfield and Davis, 1996; Munkvold *et al.*, 1999; Yates and Sparks, 2008).

Aspergillus ear rot (AER)

AER, caused by *A. flavus*, is the most important ear rot disease of maize in eastern, central and northern Africa (Siame and Nawa, 2008). The fungus is necrotrophic, destroying plant cells ahead of its infection (Smart *et al*., 1990). It overwinters as sclerotia on plant debris from the previous production season and can survive in soil. Conidia produced from sclerotia serve as primary inoculum and can be dispersed by wind and rain (Scheidegger and Payne, 2003). Infection of maize ears takes place mainly through the silk tissue after pollination (Marsh and Payne, 1984a). Senescing silks are most susceptible to infection (Marsh and Payne, 1984b) and provide entry for fungi into the ear (Hesseltine and Bothast, 1977). The fungal mycelium spreads superficially among the kernels and penetrates the kernels mainly through the pericarp. Disease symptoms include olive-green powdery mould while infected kernels may appear brown, shrunken and be lightweight (Fig. 2). Similar to *F. verticillioides*, *A. flavus* can also contaminate grain without causing any visible signs of infection (Henry *et al*., 2009). Aflatoxins are sometimes produced before harvest, but mostly contaminate maize grain following storage of maize ears in humid environments in the tropics.

Other ear rot diseases of maize

GER, caused by *F. graminearum* s.l., is characterised by a reddish-white mold that usually begins at the tip of the ear and can grow to cover the entire ear (Fig. 3) (Sutton, 1982). Mycelial growth becomes intensely pink or red and results in yield and grain quality reduction (Logrieco *et al*., 2002). *Fusarium graminearum* infects maize plants systemically from seeds, and through wounds on the stems and ears. Kernel infection through the silks is, however, the most common means of infection (Koehler, 1942). Natural epidemics of GER are often localized and sporadic, making them difficult to predict (Viger *et al*., 2001). *Fusarium graminearum* produces several economically important mycotoxins, including trichothecenes and zealeranone.

Stenocarpella maydis and *S. macrospora* (Earl) Sutton cause Diplodia ear rot (DER) of maize. Symptoms can be seen during early ear development, resulting in yellowing and drying of infected ear bracts on green plants (Flett *et al*., 1998). Infection typically starts as a dense white to greyish mold growing from the base of the ear and may cover the entire ear (Fig. 4). In addition, *S. maydis* produces fruiting bodies (pycnidia), which appear as raised black bumps that may be scattered on the husks, ears and sides of rotten kernels (Lamprecht *et al*., 2011). Latent infection of *S. maydis* shows no apparent symptoms, but when the ears are broken in half and the kernels removed, pycnidia can be found on the kernels with discoloured embryos (Nowell, 1997).

Factors affecting ear rot development and mycotoxin contamination

Environmental factors play a significant role in the development of maize ear rot. FER is most common in maize fields following hot (>28^{o}C), dry conditions (Shelby *et al*. 1994; Miller *et al*. 1995; Pascale *et al*. 2002), while *A. flavus* can grow at very high temperatures (up to 48°C) and at low water potentials (-35 MPa) (Klich *et al*., 1994). Tropical conditions, such as high temperatures and moisture, unseasonal monsoon rains during harvest and flash floods lead to fungal proliferation (Bhat and Vasanthi, 2003). Furthermore, drought, host-plant genotype and insect activity are important in determining pre-harvest contamination (Warfield and Davis, 1996; Munkvold *et al.*, 1999; Miller, 2001). The severity of FER and fumonisin contamination has been associated with insect damage (Warfield and Davis 1996; Sobek and Munkvold 1999; Munkvold and Hellmich 2000; Maiorano *et al*. 2009) and mechanical damage during harvesting (Munkvold 2003b). Insects that feed on maize ears in the field predispose kernels to fungal infection, while storage insect pests open the kernels to fungal invasion (Avantaggio *et al*., 2002). For this reason insect damage of maize is a good predictor of potential mycotoxin contamination and can serve as an early warning sign (Wagacha and Muthomi, 2008). Improper storage of dried maize may facilitate the contamination of maize with mycotoxins (Azziz-Baumgartener *et al*., 2005). Maize in Africa is commonly stored in homes and huts during times of excessive heat and humidity, which provide the ideal conditions for fungal proliferation and mycotoxin production. Additionally, poor aeration in the houses and dirty floors may promote fungal growth on wet maize kernels.

MYCOTOXINS

Mycotoxins are secondary metabolites produced by fungi in food crops. Ingestion of contaminated food is often detrimental to both human and animal health, and can result in mycotoxicosis (Murphy *et al.,* 2006; Wu, 2006; Balazs and Schepers, 2007). The two groups of mycotoxins most commonly associated with maize in Africa are the fumonisins and aflatoxins. Others include the trichothecenes, zearelanone and ochratoxins.

Fumonisins

Fumonisins are produced by a number of morphologically-related *Fusarium* species, including *F. verticillioides*, *F. proliferatum*, *F. napiforme* Marasas, Nelson & Rabie, *F. anthophilum* (A. Braun) Wollenweber, *F. dlamini* Marasas, Nelson & Toussoun*, F. nygamai* Burgess & Trimboli*, F. thapsinum* Klittich, Leslie, Nelson & Marasas and *F. globosum* Rheeder, Marasas & Nelson (Leslie and Summerell, 2006). Its production in field maize, however, is often positively correlated with the occurrence of *F. verticillioides* and *F. proliferatum*, which

predominate during the late stages of plant maturity (Warfield and Gilchrist, 1999). The reason for fumonisin production by *F. verticillioides* in maize ears is not clear, but it may provide a competitive advantage during stressful environmental conditions, such as water- and nutrient deficiency (Picot *et al*., 2010). Furthermore, fumonisin B_2 can also be produced by *Aspergillus niger* Tiegh, a fungus considered of industrially importance (Frisvad *et al*., 2007).

Fumonisins were first isolated from maize in the Eastern Cape Province of South Africa by Gelderblom *et al*. (1988). They consist of a group of structurally related polyketide-derived mycotoxins, and are classified as fumonisin B (FB) or fumonisin C (FC), based on the presence (FB) or absence (FC) of a terminal methyl group derived from an amino acid (Musser and Plattner, 1997; Sewram *et al*., 2005). The B-series fumonisins (FB_1, FB_2, FB_3 and FB_4) are most abundant in maize and maize-based food and feed. In maize grain, the incidence of FB_1 usually constitutes approximately 70%, while FB_2 and FB_3 could constitute 10-20% of the total fumonisin content (Nelson *et al*., 1993). Fumonisins have been found in almost all maize fields surveyed in Africa (Bankole *et al*., 2006).

Fumonisins have been associated with a number of human and animal diseases worldwide. The International Agency for Research on Cancer (IARC) working group on carcinogenicity risks for humans has therefore classified FB_1 as possibly carcinogenic to humans (Group 2B). Despite the prevalent occurrence of fumonisins in maize, often in very high concentrations, there are no confirmed records of acute fumonisin toxicity in humans. Fumonisins have, however, been statistically associated with oesophageal cancer in South Africa, China, Iran and Italy (Franceshi *et al*., 1990; Rheeder *et al*., 1992; Shephard *et al*., 2000; Li *et al*., 2001), and implicated in neural tube defects in new-born babies (Missmer *et al*., 2006). In animals they are known to cause equine leukoencephalomalacia (a fatal brain disease) and porcine pulmonary oedema syndrome (swelling of lungs and thorax) (Kellerman *et al*., 1990). Fumonisin B_1 is hepatotoxic in most animal species tested, including rats, horses, mice, rabbits and pigs (Gelderblom *et al*., 1994; Osuchowski *et al*., 2005). Embryotoxicity and teratogenicity were observed concurrent with or subsequent to maternal toxicity. Fumonisins are nephrotoxic in male pigs, rats, sheep, mice and rabbits, and elevated cholesterol levels in the blood of several experimentally animal species. FB_1 also induced liver cancer in rats following chronic toxic hepatitis (Gelderblom *et al*., 1994).

Aflatoxins

Aflatoxins are a family of structurally related polyketides produced by strains of *A. flavus* and *A. parasiticus* Speare in grains, nuts and dried fruit (Villers, 2014). Of the two fungal species, *A. flavus* is most commonly associated with maize grain. Seventeen aflatoxins have been described of which four are well-known and extensively studied (WHO, 1979). These include aflatoxin B_1, B_2, G_1 and G_2 (Pitt *et al*., 1993; 1994). The “B” and “G” designations refer to the blue and green fluorescent colours displayed under long-wave ultraviolet light in physico-

chemical assays, while the subscript numbers 1 and 2 indicate chromatographic mobility. Of the aflatoxins, AFB_1 often constitutes 60-80% of contaminated samples. Two other aflatoxins, described as M_1 and M_2, are metabolites of AFB_1 that occur in milk of humans and animals consuming aflatoxin-contaminated grain.

The International Agency for Research on Cancer (IARC) has classified AFB_1 and mixtures of aflatoxins as Group 1 carcinogens, which are considered as highly poisonous toxic substances (IARC, 2002). Acute exposure to aflatoxins can lead to growth impairment, childhood stunting as well as jaundice and, in severe cases, death (Gong *et al*., 2002). The most serious outbreak of aflatoxicosis occurred in Kenya where 215 deaths were reported in 2004 (Lewis *et al*., 2005). The consumption of aflatoxin-contaminated food by people infected with hepatitis B and C, common in sub-Saharan Africa, increases the risk of liver cancer by more than ten-fold compared to people with only one of the diseases (Turner *et al*., 2003). Aflatoxin exposure may also contribute to kwashiorkor (Ramjee *et al*., 1992) and impaired growth of children (Gong *et al*., 2003; Egal *et al*., 2005). An interaction between chronic mycotoxin exposure and malnutrition, immuno-suppression, impaired growth, and diseases such as malaria and HIV/AIDS has also been suggested (Gong *et al*., 2003; 2004). Other general signs of aflatoxicosis include oedema of the lower extremities, abdominal pain and vomiting (Wagacha and Muthomi, 2008). Aflatoxins in animal feed have led to liver necrosis, oxidative stress and haemorrhage in broiler chickens, pigs and cattle (Eraslan *et al*., 2005).

Other mycotoxins produced in maize grain

Fusarium graminearum produces one of three strain-specific TCT-B profiles (chemotypes), namely nivalenol (NIV) and its acetylated derivatives (NIV chemotype), deoxynivalenol (DON) and the acetylated derivative 3-acetyldeoxynivalenol (3-ADON chemotype) and DON and the acetylated derivative 15-acetyldeoxynivalenol (15-ADON chemotype) (Ward *et al*., 2002). Contaminated grain fed to livestock (especially swine) induces vomiting, feed refusal, and decreased weight gain (Christensen and Meronuck 1986). Another mycotoxin produced by *F. graminearum*, called zearalenone (ZEA) causes birth defects in exposed ruminants (Mirocha *et al*., 1976).

The ability of *S. maydis* to produce diplonine, a neurotoxin that causes symptoms similar to those caused by diplodiosis, was demonstrated by Snyman *et al.* (2011). Diplodiosis is associated with nervous system defects and neonatal losses in livestock, as well as acute toxicity in ducklings and chickens (Kellerman *et al*., 1985; Rabie *et al*., 1987; Kellerman *et al*., 1991). The potential detrimental effects in humans of diplonine, however, have not yet been demonstrated (Barros *et al*., 2008; Snyman *et al*., 2011).

Regulation of mycotoxins

More than 100 countries have established mycotoxin regulations, including 15 African countries (Van Egmond, 2002; Barug *et al*., 2003; Fellinger, 2006). The United States Food and Drug Administration (FDA) has set guidelines of 2 milligrams per kilogram (mg kg^{-1}) (FB_1) for milled, and 4 mg kg^{-1} for whole or partially milled maize products for human consumption (FDA, 2000). The European Union allows 4 mg kg^{-1} FB_1 in unprocessed maize, 1 mg kg^{-1} in processed maize and maize-based products for consumption by adults, and 0.2 mg kg^{-1} for consumption by infants and young children (European Commission, 2007). Aflatoxin contamination allowed in human foods are 4-30 parts per billion (ppb), depending on the country involved (Henry *et al*., 1999; FDA, 2004). In the US, 20 ppb is the maximum aflatoxin residue limit allowed in food for human consumption, except for milk (FAO, 1996; Wu, 2006), while 4 ppb is the maximum acceptable limits in the EU (EC, 2006; Wu, 2006). These regulations have significant implications for the production and selling of African maize and maize-based products nationally and internationally. In South Africa, the maximum tolerable level for aflatoxin levels in foods is 10 ppb (Government Gazette, 2004), but no legislation exists for fumonisins in maize and maize-based products (Shephard, 2008; Wagacha and Muthombi, 2008). Regulatory infrastructure, however, does not enable inspection and enforcement (Warburton and Williams, 2014), making the regulatory control of mycotoxins in Africa largely ineffective (Strosnider *et al*., 2006).

MANAGEMENT OF MAIZE EAR ROT DISEASE

Managing ear rot pathogens and their mycotoxins in maize requires a proper understanding of the biology, epidemiology and genetics/genomics of the mycotoxigenic fungus and host plant. For instance, *F. verticillioides* and *A. flavus* both colonise maize ears in the field, but fumonisins are produced mainly in the field before harvest, while aflatoxins are most often produced during storage. Management, therefore, should be performed both before and after the harvesting of maize grain.

Pre-harvest management strategies

Cultural control: The primary objective of cultural control of mycotoxigenic fungi is to minimize factors that result in plant stress. Extended periods of heat and drought stress that lead to increased fumonisin levels could be managed with proper irrigation schedules (Miller, 2001; Abbas *et al.*, 2012). Similarly, nutritional stresses in the field can be relieved by proper fertilisation (Blandino *et al.*, 2008a). Inoculum build-up on plant residues can be reduced by effective tillage and crop rotation practices, such as the rotation of maize with non-host crops (Flett, 1993; Munkvold, 2003b; Marocco *et al*., 2008). Adhering to planting dates and planting maize plants at lower densities also reduce mycotoxin accumulation during production

(Munkvold, 2003; Blandino *et al.*, 2008b; Abbas *et al*., 2012). However, maize cultivated by means of organic agriculture does not accumulate less fumonisins than maize cultivated conventionally (Arino *et al*., 2007; de Galaretta *et al*., 2015). The harvest of maize grain should also be carried out as close to physiological maturity as possible to prevent fumonisin contamination (Bush *et al.*, 2004).

Chemical control: Fungicides are neither effective in reducing *F. verticillioides* infection / fumonisin accumulation, nor *A. flavus* infection / aflatoxin accumulation in maize. This may be due to the husks that cover maize kernels (Nel *et al.*, 2003; Janse van Rensburg, 2012). Fumonisins were, however, reduced by 95% *in vitro* when four fungicides and a biocontrol bacterium (Serenade, *B. subtilis*) were evaluated for the control of *F. verticillioides* and *A. flavus* (Formenti *et al*., 2012). No registered fungicides are available for the control of either *F. verticillioides* or *A. flavus* in any African country. Insecticides can prevent insect wounds that contribute to fungal infection and fumonisin accumulation in maize kernels (Parsons and Munkvold, 2010).

Biological control: The use of biological control agents to manage mycotoxigenic fungi has been reported. Desjardins *et al*. (1998) found that atoxigenic *F. verticillioides* strains competitively excluded fumonisin-producing strains and prevented them from producing fumonisins. When these strains were applied by themselves through the silk channel, however, they resulted in high levels of FER. Luongo *et al*. (2005) also observed effective control of toxigenic *F. verticillioides* and *F. proliferatum* by non-toxigenic *Fusarium* species in maize residues. Most success, however, has been achieved with the use of atoxigenic strains of *A. flavus* to control toxigenic *A. flavus* and *A. parasiticus*. When introduced into the soil, these atoxigenic strains have reduced aflatoxin contamination of peanuts in the USA by 74.3-99.9% (Dorner *et al*., 1998). Atoxigenic *A. flavus* strains are now widely used to control aflatoxins of maize in several African countries (www.aflatoxinpartnership.org). Endophytic bacteria have been reported to control fumonisin-producing fungi by competitive exclusion (Bacon *et al*., 2001), while *Trichoderma* strains controlled them through competition for nutrients and space, fungistasis, antibiosis, rhizosphere modification, mycoparasitism, biofertilization and the stimulation of plant-defence mechanisms (Benítez *et al*., 2004).

Disease resistance

The planting of disease resistant plants is an effective, affordable and environmentally sound strategy to control ear rot diseases and mycotoxin accumulation (Munkvold and Desjardins, 1997). Commercial hybrids differ in their ability to accumulate fumonisins (Munkfold and Desjardins, 1997), while hybrids grown outside of their adapted range accumulate more mycotoxins than those grown inside their adapted range (Shelby *et al*., 1994). None of the

maize cultivars grown commercially in South Africa is resistant to FER and fumonisin accumulation (Rheeder *et al*., 1990; Janse van Rensburg, *et al*., 2015). In a study by Schjøth *et al*. (2008), two of the 20 Zambian hybrids evaluated accumulated less than 5.0 µg/g fumonisins across years in trials employing natural infection and artificial inoculation. A few South African breeding lines have been characterised as resistant (Small *et al*., 2012; Mouton, 2014), while Afolabi *et al*. (2007) identified five tropical inbred lines with resistance to *F. verticillioides* and fumonisin accumulation. A number of AER/aflatoxin-resistant lines, containing tropical germplasm, have been registered (Mcmillian *et al*., 1993; Betran *et al*., 2002; Williams and Windham, 2001; 2006) with recently released lines showing improved plant type and resistance (Mayfield *et al*., 2012; Williams and Windham, 2012). Genetically modified maize, expressing *Bacillus thuringiensis* genes (BT maize), have been found to accumulate less fumonisins than their non-modified isolines (Munkvold *et al*., 1999).

Postharvest management strategies

Poor harvesting practises, improper storage and less than optimal conditions during transportation, marketing and processing can contribute to fungal growth and mycotoxin production in maize (Wagacha and Muthomi, 2008). Attempts to detoxify fumonisins by chemical methods and heat treatment have been met with limited success as these mycotoxins are heat-stable (Humpf and Voss, 2004). It is thus important to remove infected kernels during grain handling to prevent fungal development and fumonisin accumulation after harvest (Sydenham *et al.*, 1994; Munkvold and Desjardins, 1997). The sorting, winnowing, washing and crushing, combined with dehulling of maize grains were effective in achieving significant aflatoxin and fumonisin removal (Fandohan *et al*., 2005b, van der Westhuizen *et al*., 2011a). In a study by van der Westhuizen *et al*. (2011b) the positive impact of washing and sorting of maize kernels on fumonisin exposure of Africans in the Eastern Cape Province, South Africa was demonstrated. However, removing infected kernels may not be entirely effective, as fumonisins can still be present in visibly healthy kernels (Clements *et al*., 2004; Small *et al*., 2012).

Maize should be stored under clean, dry and cool conditions (ideally 1-4˚C), and protected from insects, rodents and birds. Biocides can be used to control insects and fungi during storage (Munkvold, 2003b). In a study by Hell *et al*. (2000), storage or cotton insecticides, mechanical means such as sorting and smoke protected maize against pests. The cleaning of stores before loading them with the new harvest also led to lower aflatoxin levels. Fewer aflatoxins were found when maize was stored in an "Ago" made from bamboo or when bags were used as secondary storage containers, compared to the aflatoxin of maize stored in a “Secco”, a giant basket made from Happharhenia diplandra or “Zingo”, a granary with a wooden conical base (Hell *et al*., 2000). The use of clay and surface-modified clay additives to reduce aflatoxins in food and feed has also been reported (Philips *et al*.,

2008; Jaynes and Zartman, 2011). Camou-Arriola and Price (1989) found that heating to 121^{o}C and alkaline treatment of naturally contaminated maize prior to frying resulted in very low levels of chemically detectable aflatoxin.

RESISTANCE IN MAIZE TO EAR ROT PATHOGENS AND THEIR MYCOTOXINS

Host-plant resistance is the most effective means to reduce ear rot diseases and minimise the risk of mycotoxin accumulation in maize (Munkvold, 2003b; Afolabi *et al.*, 2007). Maize inbred lines resistant to FER and AER have been reported (King and Scott, 1981; Nankam and Pataky, 1996; Clements *et al*., 2004), but none of these lines were immune. Resistance to ear rot pathogens, therefore, appears to be quantitative rather than qualitative. In maize, plant resistance consists of two mechanisms: constitutive structural barriers and induced biochemical responses. According to Pérez-Brito *et al.* (2001) and Robertson *et al.* (2006), these mechanisms are presumably similar for resistance to FER and fumonisin accumulation, suggesting that selection for the one should accommodate the other. This argument is further supported by Robertson-Hoyt *et al*. (2006) who reported a moderate to high heritability and strong genetic correlation between FER and fumonisin resistance in two maize populations. However, Desjardins and Plattner (2000) demonstrated that naturally occurring *F. verticillioides* isolates, which differed in their ability to synthesise fumonisins, did not differ in their ability to produce FER symptoms following silk channel inoculation. Also, *F. verticillioides* mutants with inactivated *FUM*1 genes were equally capable of producing ear rot symptoms following silk channel inoculation when compared to wild type controls with normal *FUM*1 gene function (Desjardins *et al*., 2002). Desjardins *et al*. (2007) furthermore demonstrated that fumonisin-producing and non-fumonisin producing *F. verticillioides* isolates did not differ in their ability to cause seedling blight. The fumonisin-producing strain, however, was more effective in systemically colonising maize seedlings. Stumpf *et al*. (2013) reported a lack of correlation between Fusarium-damaged kernels, fungal incidence in grain and fumonisin levels, while Mouton (2014) reported a significant though poor correlation between FER severity and fumonisins, as well as between FER severity and fungal target DNA in maize grain.

Structural resistance barriers

The first line of defence in a maize plant to *F. verticillioides* infection involves structural barriers. These barriers include pericarp thickness, silk detachment and husk tightness (Headrick and Pataky, 1991; Warfield and Davis, 1996; Munkvold, 2003b). Thicker pericarps are more resistant to breaking and insect damage, thereby allowing less kernel infection by *F. verticillioides* to occur (Hoenisch and Davis, 1994). Once infected, however, the thicker pericarp could slow down kernel drying, making it suitable for fungal growth and fumonisin

production (Cao *et al*., 2014). While no correlation was reported between pericarp thickness and resistance to FER in Croatian inbred lines and hybrids (Ivic *et al*., 2008), Sampietro *et al*. (2009) found that the pericarp and its wax content were responsible for resistance to fumonisin accumulation in most genotypes assayed in Argentina.

Husk tightness also plays an important role in maize ear infection by *F. verticillioides* (Cao *et al*., 2014). Genotypes with husks that were less tight were more susceptible to kernel infection. Furthermore, silk detachment and the age of silks had been associated with resistance to *F. graminearum* (Reid *et al*., 2012). In a study by Duncan and Howard (2010) the stylar canal was shown to be an infection court for *F. verticillioides*, who assumed that its architecture (open vs close) may serve as a potential resistance barrier to fungal ingress in the absence of any other infection pathway.

Biochemical resistance mechanisms

Fumonisin production by *F. verticillioides* is regulated by physico-chemical changes taking place during kernel maturation. *In vitro* studies have shown that changes involving pH and kernel substrates (carbon, nitrogen, sugar, and starch) regulate fumonisin production by *F. verticillioides* (Marín *et al*., 1995; Jurado *et al*., 2008; Kim and Woloshuk, 2008; Schmidt-Heydt *et al.,* 2008; Picot *et al.*, 2010). As maize kernels mature the amount of water available for fungal metabolism (water activity (aw)) decreases, which induces fumonisin production by *F. verticillioides* (Jurado *et al*., 2008; Schmidt-Heydt *et al.*, 2008). Acidic pH conditions enhance FB_1 biosynthesis by *F. verticillioides*, and alkaline pH conditions repress it (Keller *et al.*, 1997; Flaherty *et al.*, 2003; Kim and Woloshuk, 2008).

High levels of pericarp phenylpropanoids (phenolics) have been associated with less FER and fumonisin accumulation in maize grain (Sampietro *et al*., 2013). Phenolics have also been shown to inhibited *Fusarium* spp. growth and mycotoxin production. Samapundo *et al*. (2005) found a decrease in the colony growth rate and increase in the lag phase duration for both *F. verticillioides* and *F. proliferatum* in response to increasing concentrations of caffeic and vanillic acid. In a study by Ferrochio *et al.* (2013) the growth rates of *F. verticillioides* and *F. proliferatum* was significantly reduced in the presence of high doses of ferulic acid. Furthermore, phenolics resulted in a reduction of DON and fumonisins produced *in vitro* by *Fusarium* species (Boutigny *et al.*, 2009).

Common resistance mechanisms to maize to ear rot pathogens and their mycotoxins

Common resistance mechanisms to maize ear rot pathogens and their mycotoxins have been reported. Reid *et al*. (2009) demonstrated that inbred lines resistant to GER had the lowest disease severity following inoculation with *Ustilago maydis* (De Candolla) Corda (common smut) and *F. verticillioides*. Genotypes with good resistance to both *F. verticillioides* and *A. flavus* were also identified by Henry *et al*. (2009). The strong, significant

correlations between these ear rot pathogens and their mycotoxins allowed Robertson-Hoyt *et al*. (2007) to conclude that resistance in maize to both species are present in the same genotype. South African inbred lines resistant to FER were also resistant to GER and DER, caused by *S. maydis*, across three localities (Mouton, 2014).

Molecular basis of resistance in maize to ear rot pathogens

Gene expression analysis and proteomic studies have been used to investigate the maize response to *F. verticillioides* and other ear rot pathogens. The transcriptional response of maize kernels to *F. verticillioides* has been well investigated by Lanubile *et al*. (2010). In a microarray-based study, they classified expressed sequences from a resistant and susceptible inbred line into 11 functional categories mostly assigned to cell rescue, defence and virulence. Defence-related genes in the resistant line were transcribed at higher levels than in the susceptible line prior to infection, while these were induced after pathogen infection in the susceptible line (Lanubile *et al*., 2010; 2012a, b). In a next-generation RNA sequencing study, similar plant responses were observed in resistant and susceptible maize genotypes following inoculation with *F. verticillioides* (Lanubile *et al*., 2014). The induction of genes involved in pathogen perception, signalling and defence, including WRKY transcription factors and jasmonate- / ethylene-mediated defence responses, however, was much greater in the resistant genotype.

Pathogenesis-related (PR) proteins were expressed at higher levels in the resistant compared to the susceptible line (Lanubile *et al*., 2012a). PR proteins are believed to restrain growth, multiplication and/or the spread of the invading pathogen (Kitajima and Sato, 1999). Sekhon *et al*. (2006) also reported on the expression of PR-genes in maize kernels following infection by *F. graminearum* and *F. verticillioides*. The PR-4 and PR-5 showed developmental as well as fungus-induced expression in maize. Zhang *et al*. (2011) identified the *FvMK*1 (mitogen-activated protein kinase) gene in *F. verticillioides*, which regulates conidiation, pathogenesis and also lowers the activity of the *FUM*1 and *FUM*8 genes. Resistance in maize to *A. flavus* also included PR proteins (β-1,3-glucanases, chitinases), ribosome inactivating proteins (RIPs) and zeamatin (Dolezal *et al*., 2014). Interestingly, Campos-Bermudez *et al.* (2013) reported no important differences in the transcriptional and metabolic profiles of resistant and susceptible maize lines following *F. verticillioides* inoculation. This suggested that preformed or constitutive defence mechanisms are primarily responsible for defence to *F. verticillioides* in maize.

ENHANCING MAIZE FOR RESISTANCE TO EAR ROT PATHOGENS

Conventional breeding

Maize breeding programmes at both public and private institutions are initiating and expanding their efforts to develop disease-resistant inbreds and hybrids (Mesterhazy *et al*., 2012). A number of international institutions like the International Maize and Wheat Improvement Center (CIMMYT) and the International Institute of Tropical Agriculture (IITA) have established breeding programmes with the primary focus on producing inbred lines with improved resistance to *A. flavus* and aflatoxins. The development of resistant cultivars, however, has been slow due to the polygenic, quantitative nature of resistance to mycotoxigenic fungi (Hart *et al*., 1984; Pérez-Brito *et al*., 2001; Robertson-Hoyt *et al*., 2006; Reid *et al*., 2009), the unavailability of immune germplasm (Pérez-Brito *et al*., 2001; Clements *et al*., 2004), and the effect of the environment on ear rot development and mycotoxin production (Shelby *et al*., 1994; de la Campa *et al*., 2005; Cao *et al*., 2014). The development of maize lines and hybrids, therefore, is a long (8-10 years) and costly process that needs to be conducted as effectively as possible. To date, no maize hybrids completely resistant to FER and fumonisin accumulation has been produced by conventional breeding (Clements and White, 2004; Eller *et al*., 2008).

Diallel analysis to determine the general and specific combinability (GCA; SCA) of resistant genotypes have been reported for *F. verticillioides* (Hung and Holland, 2012; Henry *et al*., 2009), *A. flavus* (Naidoo *et al*., 2002) and *F. graminearum* (Reid *et al*., 1992). The response of an inbred line to *F. verticillioides* and fumonisins, and the corresponding GCA in hybrids, were significantly correlated. This indicates that an efficient way to improve resistance to FER and fumonisin contamination resistances in hybrids is to first evaluate and select resistant inbred lines that can be used to develop resistant hybrids (Hung and Holland, 2012). However, hybrid performance for resistance to GER could not be predicted based on the GCA of inbred line parents (Reid *et al*., 1992). Williams and Windham (2009) evaluated lines with resistance to aflatoxin contamination for GCA and SCA for resistance to fumonisin accumulation and released two lines with resistance to fumonisins and aflatoxins. Eller *et al*. (2010) demonstrated improved resistance to FER and fumonisins in inbred lines derived from cross-pollination of resistant and elite maize lines. The subsequent hybrids produced from the crossing of improved lines with elite lines, however, did not demonstrate improved FER and fumonisin accumulation although some improved lines performed well as an inbred line and as a component of a hybrid (Eller *et al*., 2010).

Quantitative trait loci (QTL) associated with resistance to FER and fumonisin accumulation has been mapped (Pérez-Brito *et al*., 2001; Robertson-Hoyt *et al*., 2006; Ding *et al*., 2008; Li *et al*., 2011; Chen *et al*., 2012; Zila *et al*., 2013). The QTLs, however, displayed pleiotropic effects, sometimes resulting in resistance to both traits (Pérez-Brito *et*

al., 2001; Ding *et al*., 2008, Xiang *et al*., 2010). QTL analyses have also demonstrated pleiotropic effects for resistance to other mycotoxigenic fungi and/or their associated mycotoxins. In QTL studies involving multiple ear rot pathogens, maize resistant to FER and fumonisin accumulation was also resistant to *F. graminearum* and/or *A. flavus*, with common loci for ear rots and fumonisins, respectively (Pérez-Brito *et al*., 2001; Robertson-Hoyt *et al*., 2007; Xiang *et al*., 2010). Research conducted by Robertson-Hoyt *et al*. (2007) revealed that some of the genes involved in resistance to FER and AER caused by *A. flavus*, as well as their associated mycotoxins (fumonisin and aflatoxin, respectively), were identical or genetically linked. These studies implied that breeding for resistance against one type of ear rot pathogen and its mycotoxin may lead to similar responses for another ear rot pathogen and its mycotoxin.

Unconventional breeding

Genetic modification: Genetically modified crops are plants of which the DNA has been altered through the introduction of a foreign gene to express a trait not inherent to the modified plant. Three transgene-mediated strategies have been proposed for the management of FER and fumonisin accumulation in maize (Duvick, 2001). These include (i) the reduction of *F. verticillioides* infection, (ii) the degrading of fumonisins, and (iii) interfering with the fumonisin biosynthetic pathway. To reduce infection by the fungus, the incorporation of antifungal and/or resistance genes, as well as the over-expression of defence-related genes, is required. Catabolic enzymes from microbes have been used to detoxify fumonisins, both *in vitro* and *in situ*, before they accumulate in the plant (Yoneyama and Anzai, 1991; Lu *et al*., 1998; Zhang *et al*., 1999). Fumonisin esterase and amine oxidase genes encoding fumonisin-degrading enzymes have been identified in *Exophiala spinifera* de Hoog and Hasse (Duvick, 2001). None of these genes have, however, been successfully introduced into maize. Maize plants have been genetically engineered to interfere with the biosynthesis of aflatoxins and trichothecenes (Brown *et al*., 1999; Okubara *et al*., 2002), but not with that of fumonisins. The best-known example of using genetically modified maize for reducing FER and fumonisin contamination of grain is Bt maize (Munkvold *et al*., 1999; Abbas *et al*., 2013). This is due to the close association between kernel damage by insects and infection by *F. verticillioides* (Munkvold, 2003a). Bt maize plants that prevent insect damage, therefore, also reduce fumonisin contamination of maize grain. Genetically modified maize is not authorized in all countries and, consequently, conventional breeding efforts are still commonly used.

Mutation breeding: The exposure of seeds or other heritable material to chemicals or radiation with the purpose to induce changes (mutations) is known as mutation breeding. Nuclear technology for crop improvement makes use of ionizing radiation, which causes induced mutations with high mutation frequency in plants (Yadav *et al*., 2015). These

mutations might be beneficial and alter physiological characters of plants, including plant height, ear height and improved root architecture (Borzouei *et al*. 2010; Khawar *et al*. 2010). The radiation of seeds may also cause genetic variability that enables breeders to select new genotypes with improved grain yield and quality (Noreen and Ashraf 2009).

The exposure of cells to non-lethal doses of ionising radiation, such as gamma irradiation, results in gene mutations that generate variation (Ahloowalia *et al*., 2004). Ionising radiation, specifically gamma irradiation, causes single or double DNA strand breaks that can result in the deletion of single or multiple nucleotides. The subsequent repair may result in gene disruption, coding changes in genes, as well as chromosomal rearrangements (Dadachova and Casadevall, 2008). Mutation breeding has been successfully used to generate genetic variation in cereal crops, including maize, for a number of reasons including enhanced yield and productivity, altered ear length, drought tolerance and enhanced stem structure (Mashev, 1995; Jain, 2010; Tomlekova, 2010). It can thus potentially provide an attractive means for generating resistance to FER and fumonisin accumulation.

In order to evaluate the effects induced by gamma radiation, physiological changes such as seed germination and plant growth stimulation or inhibition are measured (Borzouei *et al*., 2010). Low doses of gamma have improved plant vigour, grain development and the yield of wheat (*Triticum aesticum* L.) (Singh and Datta, 2010), grapevine (*Vitis vinifera*) (Charbaji and Nabulsi, 1999) and rocket (*Eurica sativa*) (Moussa, 2006). Exposure to high doses resulted in a reduction of the germination capacity with a corresponding decline of plant growth and development of wheat (Chaomei and Yanlin, 1993). The germination process of *Echinacea purpurea* (L.) Moench was affected by high levels of irradiation and resulted in a reduction of germination capacity (Ichim *et al*., 2006), while growth of young tobacco (*Nicotiana tabaccum* L.) plants was significantly reduced at 30-50 Gy and completely stopped following exposure at 70 Gy (Cho *et al*., 2000). Studies on mung bean (*Vigna radiata* (L.) (Singh and Sharma, 1993), alfalfa (*Medicago sativa* L.) (Rejili *et al*., 2008), rice (*Oryza sativa* L.) (Shereen *et al*., 2009) and okra (*Abelmoschus esculentus* Moench) (Hegazi and Hamideldin, 2010) revealed that exposure at low doses lead to an increase of the assimilatory pigments content, while at high doses it decreases significantly as compared with non-irradiated ones.

CONCLUSION

Maize is one of the most important staple food crops produced in sub-Saharan Africa, and provides the majority of the calorie intake for more than 1 billion Africans. The production of maize on the African continent, however, is subjected to unique constraints including poor infrastructure and transport, and the lack of irrigation water, good seed and pesticides. The

contamination of maize grain with mycotoxigenic fungi and mycotoxins is a leading concern, as it poses a significant threat to food safety.

Planting maize genotypes with enhanced host-resistance is considered the most practical, affordable and environmentally sound method of managing maize ear rot pathogens and their mycotoxins in Africa. Resistance to FER and fumonisin accumulation has been identified in a small number of locally adapted breeding material (Small *et al*., 2012; Mouton, 2014), but such resistance needs to be introduced in high-yielding and locally adapted hybrids. As yet, conventional breeding has not been able to introgress disease resistance into commercial maize cultivars. Understanding host resistance at a molecular level, however, could provide additional opportunities for introducing resistance into maize cultivars by means of molecular breeding and genetic engineering.

The objective of **Chapter 2** will be to evaluate elite inbred lines developed for commercial purposes and used as high yielding female lines, for resistance to FER, *F. verticillioides* colonisation and fumonisin accumulation across several environments over two seasons. Previously characterised inbred lines will be included in the evaluation, and the stability of resistance in inbred lines will be determined. Based on the current indications of common resistance to *F. verticillioides*, *A. flavus* and their associated mycotoxins, AER/aflatoxin-resistant inbred lines obtained from CIMMYT-Kenya will be evaluated for resistance to FER, *F. verticillioides* colonisation and fumonisin accumulation under South African and Kenyan conditions in **Chapter 3**. To determine the efficacy of gamma irradiation to generate resistance to FER and fumonisin contamination, elite maize inbred lines will be mutated and interbred for 4 years in **Chapter 4**. The value of selecting for reduced FER severity, following artificial inoculation, in obtaining *F. verticillioides* / fumonisin-resistant lines will also be determined during mutation breeding, as visual disease assessment will be used as the selection criteria. The molecular response of maize inoculated with *F. verticillioides* will be determined during the early colonisation period (0–7 days after inoculation) by next-generation RNA sequencing in **Chapter 5**. This will provide insight on the *F. verticillioides*-maize interaction beyond the initial infection and colonisation timeframe currently available in literature. Knowledge on induced and/or sustained plant defence mechanisms to fungal infection could lead to novel management strategies of FER and fumonisin accumulation in maize.

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Figure 1. Fusarium ear rot of maize characterised by white fungal growth on individual or clusters of kernels and distributed randomly across the maize ear (photo: W.F.O Marasas).

Figure 2. Aspergillus ear rot characterised by olive-green powdery mould on maize kernels (www.apsnet.org).

Figure 3. Gibberella ear rot of maize identified by the spread of pink or purple mycelial growth from the tip of the maize ear (Photo: I. Beukes).

Figure 4. Diplodia ear rot of maize characterised by dense white/grey mycelia commencing from the ear base to the tip of the maize ear (Photo: I. Beukes).

CHAPTER 2

Multi-environment evaluation of maize inbred lines for resistance to *Fusarium verticillioides* and fumonisin accumulation[1]

ABSTRACT

Fusarium verticillioides is a fungus commonly associated with maize around the world. It causes Fusarium ear rot (FER), which significantly affects grain yield and quality due to the moulding of kernels, and contaminates grain with a group of mycotoxins called fumonisins. Host-plant resistance is considered the most sustainable means to manage FER and fumonisin contamination of grain. The improvement of maize for disease resistance is dependent on the identification of resistant elite breeding material. Therefore, the aim of this study was to evaluate 18 South African inbred lines for resistance to *F. verticillioides* and fumonisin accumulation at five localities over 2 years. Maize ears, artificially inoculated with *F. verticillioides* under field conditions, were visually assessed for FER symptoms. Thereafter, *F. verticillioides* colonisation and fumonisin content was determined by quantitative real-time PCR and liquid chromatography tandem mass spectrometry, respectively. Significant genotype x environment interactions were found for the 2010/11 and 2011/12 seasons. Inbred lines CML 390, RO 424W, US 2540W and VO 617y-2 showed low FER severity (≤5%), fungal target DNA (≤0.1 ng μL^{-1}) and fumonisins levels (≤5 mg kg^{-1}) at most test locations. Genotype main effect and genotype x environment (GGE) biplots showed that inbred lines CML 390, US 2540W, RO 424W were most resistant to FER, fungal colonisation and fumonisin accumulation, respectively, while inbred line RO 424W was most stable across environments. The identified lines could serve as valuable sources of resistance against *F. verticillioides* and its fumonisins in local breeding programmes. Their use in genomic and proteomic studies could further contribute to better understand the molecular basis of resistance to *F. verticillioides* and fumonisin accumulation in maize.

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INTRODUCTION

The fungus *Fusarium verticillioides* (Sacc.) Nirenberg is commonly associated with maize (*Zea mays* L.) (Kommedahl and Windels, 1981). It infects plants at all developmental stages and can cause numerous diseases including Fusarium ear rot (FER), root and stalk rot (Kommedahl and Windels, 1981; Munkvold *et al*., 1997). *Fusarium verticillioides* can also colonise vegetative and reproductive tissues without showing any disease symptoms (Foley, 1962; Bacon and Hinton, 1996; Munkvold *et al*., 1997). Silks and wounds caused by insects are considered the most common infection pathways (Smeltzer, 1959; Headrick and Pataky, 1991; Munkvold and Carlton, 1997; Munkvold *et al*., 1997), while systemic infection through seeds and roots has also been demonstrated (Munkvold *et al*., 1997; Oren *et al*., 2003; Wu *et al*., 2011). The subsequent development of FER reduces both the yield and quality of maize, and can result in economic losses.

Fusarium verticillioides can exist as an endophyte in maize plants and may become pathogenic under conditions which are poorly understood (Munkvold *et al*., 1997; Bacon *et al*., 2008; Cao *et al*., 2014). The fungus also produces toxic secondary metabolites known as mycotoxins and, in particular, fumonisins (Gelderblom *et al*., 1988). The role of fumonisins in the pathogenesis of *F. verticillioides* is a topical issue, however, several researchers have shown that fumonisins are not required to cause seedling blight or FER, but contributes to the severity of these (Desjardins and Plattner, 2000; Desjardins *et al*., 2002; Desjardins *et al*., 2007). In contrast, Glenn *et al*. (2006) and Williams *et al*. (2007) demonstrated that only fumonisin-producing *F. verticillioides* strains were able to cause foliar disease symptoms on seedlings of a sweet maize hybrid. Fumonisins have been found in developing kernels (R2 blister stage) but tend to accumulate in the later stages of plant development as early harvest of maize plants have resulted in significantly less fumonisin contamination (Bush *et al*., 2004; Blandino *et al*., 2009; Parsons and Munkvold, 2010; Picot *et al*., 2011).

Fumonisins consist of a family of at least 28 analogues, of which fumonisins B_1, B_2 and B_3 are the most common. Fumonisin B_1 has been associated with human diseases such as oesophageal cancer (Marasas *et al*., 1981; Franceshi *et al*., 1990; Rheeder *et al*., 1992), neural tube defects in new-born babies (Missmer *et al*., 2006) and predisposition to HIV (Williams *et al*., 2010). In animals it causes leukoencephalomalacia in horses (Marasas *et al*., 1988; Kellerman *et al*., 1990), liver cancer in rats (Gelderblom *et al*., 1994) and neuro-degeneration in mice (Osuchowski *et al*., 2005). The prevalence of fumonisins in maize and maize-based products intended for human and animal consumption has resulted in many countries introducing maximum tolerable levels for the toxin (Bolger *et al*., 2001; CFSAN 2001). South Africa, however, has no legislation on the maximum tolerable limit of fumonisin in maize and maize-based products, despite fumonisin levels in rural areas exceeding

tolerable limits enforced in Europe and USA (Shephard *et al*., 2007; Shephard 2008; Ncube *et al*., 2011).

An affordable and environmentally sound manner to reduce high fumonisin levels in food and feed is by planting resistant, locally adapted maize cultivars. The polygenic, quantitative nature of resistance to FER and fumonisin accumulation, however, makes the improvement of maize for resistance to *F. verticillioides* particularly challenging. Quantitative trait loci identified by Pérez-Brito *et al*. (2001) and Robertson-Hoyt *et al*. (2006) have had relatively small effects on resistance and are inconsistent between maize populations of different genetic backgrounds. Older or exotic germplasm may serve as a source of resistance but generally lack desirable agronomic traits (Clements *and White,* 2004; Eller *et al.,* 2008, 2010). Breeders are thus challenged with introducing polygenic resistance alleles, linked to polygenic alleles for inferior agronomic traits, into elite breeding gene pools from unadapted lines (Zila *et al*., 2013).

The development of FER is strongly influenced by genotype x environment interactions (GEI), whereas fumonisin production is associated with environmental conditions such as hot, dry weather, followed by periods of high humidity (Shelby *et al.,* 1994; de la Campa *et al*., 2005; Cao *et al*., 2014). Inbred lines exhibiting resistance at one locality often respond differently when tested in a another environment due to GEI (Shelby *et al*., 1994; Robertson-Hoyt *et al*., 2006; Small *et al*., 2012). The stability of genotype response is an important consideration to ensure the commercial success of a cultivar. Stability may be defined as the consistency of a genotype to perform well across environments, despite being affected by the GEI (Riaz *et al*., 2013). Maize genotypes grown outside of their normal production areas were likely to be more susceptible to FER and fumonisin contamination (Shelby *et al*., 1994). However, Robertson *et al*. (2006) determined that differences in genotypes, rather than a lack of correlation between genotype performances at different environments, primarily contributed to significant genotype by environment (G x E) effects. Thus, determining the response of inbred lines to FER, fungal and fumonisin contamination across different environments is vital to determine which factors (G, E or GEI) affect disease development and fumonisin accumulation. Breeders currently select for reduced FER to achieve reduced fumonisins, even though studies have indicated that the correlation between FER and fungal target DNA is often poor (Afolabi *et al*., 2006; Clements *et al*., 2004; Small *et al*., 2012, Janse van Rensburg *et al*., 2015).

A small number of maize inbred lines resistant to FER and fumonisin accumulation have recently been identified in South Africa (Small *et al*., 2012). The first objective of this study was to evaluate these and seven additional South African maize inbred lines for resistance in multi-location trials over 2 years. The second objective was to determine whether the inbred lines respond similarly to FER, fumonisin accumulation and mycotoxin contamination across environments.

MATERIALS AND METHODS

Plant material evaluated

Eighteen maize inbred lines selected by plant breeders at the Agricultural Research Council – Grain Crops Institute (ARC-GCI) in Potchefstroom, South Africa were evaluated for resistance to FER, fungal colonisation and fumonisin accumulation (Table 1). These included seven elite maize inbred lines with excellent yield potential, but whose status to FER and fumonisin contamination was unknown. Inbred lines previously characterised as resistant, intermediately resistant and susceptible to FER and fumonisin accumulation in two localities (Small *et al*., 2012) were also included in the study. All inbred lines had superior agronomical traits and represented a diverse genetic background (Table 1).

Field localities

Maize inbred lines were planted at five localities, each differing in macro- and micro-climatic conditions, during the 2010/11 and 2011/12 growing seasons. Weather data, including the maximum temperature, rainfall and humidity for December to June of each year, was obtained from the ARC's weather stations located at each respective locality. The field locations included Buffelsvallei (grid ref.: 26˚48'S, 26˚61'E; altitude, 1 383 m) and Potchefstroom (grid ref.: 26˚73'S, 27˚07'E; altitude, 1 349 m) in the Northwest province; Vaalharts (grid ref.: 27˚95'S, 24˚83'E; altitude, 1 180 m) in the Northern Cape province; and Cedara (grid ref.: 29˚54'S, 30˚26'E; altitude, 1 068 m) and Makhatini (grid ref.: 22˚39'S, 32˚17'E; altitude, 77 m) in the KwaZulu-Natal province (Fig. 1).

Standard procedures to prepare fields were followed at all the trial locations. Maize kernels were hand-planted (two seeds per hill) in double-row 10-m plots, with an intra-row spacing of 0.3 m and an inter-row spacing of 1 m. The trials were planted using a randomised complete block design and replicated three times, with experimental plots thinned to 33 plants per plot 3 weeks after emergence. The Potchefstroom, Buffelsvallei and Makhatini trials were conducted under dryland conditions while the Potchefstroom and Makhatini trials were supplemented with overhead irrigation when required. The Cedara trials were irrigated with a central pivot system, whereas the Vaalharts trials were flood irrigated on a weekly basis.

Fungal isolates and inoculum production

Well-characterised isolates of *F. verticillioides*, collected in the same production areas as the trial locations, were used in this study. Isolates GCI 315 and GCI 790 were collected from infected maize in Ndwedwe (KwaZulu-Natal) and Rushof (Northern Cape), respectively, and MRC 826 were collected in the former Transkei (Eastern Cape). Conidial suspensions of each isolate were produced by inoculating mycelia into Armstrong medium (Booth, 1971) and

incubating the suspensions in a shaker-incubator for 3-4 days at 25ºC at 100 revolutions min^{-1} (rpm). Following incubation the spores were separated from the mycelia by filtration using double-layered sterile cheesecloth, and concentrated by centrifugation at 2 465 G force (G) for 10 min. The conidial pellet was washed twice with deionized, autoclaved H_2O of volumes equivalent to that of the original spore suspension. The microconidial concentration was determined using a haemocytometer, and adjusted to a final concentration of 1 x 10^6 conidia ml^{-1}. Equal volumes of the three conidial suspensions were combined to produce the final inoculum. The inoculum was kept at 4°C until inoculation, and spore viability confirmed by plating onto PDA immediately after the field inoculation.

Artificial inoculation and visual assessment of disease severity

The primary ear of each plant was inoculated 14 days after silking by injecting a conidial cocktail (2 ml) down the silk channel with a sterile Terumo needle (18 G × 1.5") and syringe (Afolabi *et al*., 2007). Once the grain dried (12 to 18% moisture), the inoculated ears were manually harvested, de-husked and subjected to visual assessment for FER symptoms. Disease severity was determined by estimating the percentage of each ear covered by visible symptoms of infection (Clements and White 2004). The maize ears were then dried to 12% moisture, mechanically shelled and the kernels bulked per trial plot. A 250-g kernel sample was taken from each bulked grain sample and ground to flour using a Cyclotech sample mill (Foss Tecator, Hoganas, Sweden). The mill was thoroughly cleaned with high pressure air between samples to avoid cross contamination. Flour samples were stored at -20°C until the extraction of fumonisins and genomic DNA was performed.

Quantification of *Fusarium verticillioides* in maize grain

DNA was extracted from fungal cultures and 2-g milled maize samples according to Boutigny *et al*. (2012). Fungal cultures were grown in 100 ml potato dextrose broth (PDB) and incubated at 25°C on a rotary shaker at 100 revolutions min^{-1} (rpm). After 2 weeks the mycelium was harvested by filtration through double layered, sterile cheesecloth, rinsed twice with autoclaved distilled water and freeze-died. The freeze-dried mycelia of MRC 826, GCI 315 and GCI 790 were stored at -20°C until genomic DNA was extracted. The DNA isolation for both fungal and maize samples was initiated with a CTAB/PVP lysis, followed by an extraction step using a DNeasy® Plant Mini kit (QIAGEN) according to the manufacturer's instructions. An additional phenol purification was performed on fungal samples before commencing with the commercial extraction kit (Boutigny *et al*., 2012). The quantity and purity of the DNA was determined with a NanoDrop ND-1000 spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). The DNA was then diluted to a concentration of 10 ng μL^{-1} and stored at -20°C.

The absolute quantification of *F. verticillioides* in maize samples was determined as follows: A standard curve was first prepared by diluting DNA of MRC 826 (16.1 ng μL^{-1}) 2^3, 2^4, 2^5, 2^6 and 2^7-fold in maize DNA that was free of fungal contamination (10 ng μl^{-1}). The standard curve had a slope of between -3.1 and -3.6 and linearity above 0.98. The ΔCt between extrapolated and measured Ct number of the undiluted sample was <0.5 (CRL-EM-01/ 08, 2008). Regression equations of standard curves were highly significant ($R^2 > 0.99$).

Fungal target DNA in maize samples was determined by quantitative real-time polymerase chain reaction (qPCR) according to the method described by Boutigny *et al*. (2012). The sensitivity of the qPCR assay to detect isolates GCI 315 and GCI 790 was determined prior to the quantification of *F. verticillioides* in maize samples. DNA from maize samples was thereafter analysed in duplicate, while standard pathogen DNA (2^3-fold dilution of pathogen DNA in clean maize DNA) and DNA-free water were included as positive and no template controls, respectively.

Fumonisin content determination

Fumonisins were extracted from 5-g milled maize samples as follows: Twenty ml methanol extraction buffer (MeOH/HPLC grade water; 70:30 v/v) was added to each sample and the suspension shaken at 200 rpm in an incubator/shaker set at 25^{o}C for 30 min. The samples were then centrifuged at 500 G at 4^{o}C for 10 min. A sterile syringe was used to remove ±2 ml supernatant, which was filtered through a 0.20-µm recombinant cellulose (RC) filter into a 2-ml Eppendorf tube. The samples were kept at 4^{o}C overnight after which they were centrifuged for 10 min at 17.2 G before they were transferred to LC-MS/MS glass vials. Samples for fumonisin analysis were diluted in a 1:1 ratio with HPLC-grade water and sent to the Central Analytical Facility (CAF) at Stellenbosch University for the quantification of FB_1, FB_2 and FB_3.

Fumonisin contamination of maize samples was determined by a dilute-and-shoot method using liquid chromatographic tandem mass spectrometry (LC-MS/MS). It was performed on a Quattro Micro triple quadrupole mass spectrometer from Waters/Micromass (Manchester, UK), equipped with an Alliance 2695 HPLC system (Waters) and Mass Lynx NT software 4.1 for data acquisition and processing, respectively. The electrospray ionization (ESI) source was used in the positive mode and the settings were optimized for the best sensitivity. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with argon employed as collision gas and a cone voltage of 25 V was used for all transitions.

Fumonisin standards (B_1 [10 mg], B_2 [10 mg], and B_3 [1 mg]), guaranteed 95% pure, were obtained from the Medical Research Council's Programme on Mycotoxins and Experimental Carcinogenesis (MRC-PROMEC), Tygerberg, South Africa. Reconstituted standards were utilised in a dilution series that ranged between 0.05 and 20 mg kg^{-1} for FB_1

and FB_2, and between 0.005 and 2 mg kg^{-1} for FB_3. A calibration curve was performed with each set of samples analysed, and a non-toxin control represented by pure MeOH/HPLC grade water (70:30) was included. Each standard and sample (5 µl) was injected into the LC-MS/MS system, and samples with results above the calibration curve limits were diluted and re-analysed. After the volume of extract used in the purification procedure was adjusted, the minimum limits of quantification for FB_1, FB_2 and FB_3 were 0.02 mg kg^{-1}, 0.002 mg kg^{-1} and 0.02 mg kg^{-1}, respectively.

Masslynx processing software was used to integrate peak areas and quantify the amount of FB_1, FB_2 and FB_3 in maize grain in micrograms. The concentration of FB_1, FB_2 and FB_3 in the maize sample was calculated as follows:

C (mg kg-1) = (A × D)/(W)
C = concentration of fumonisin in maize sample (mg kg^{-1})
A = concentration obtained from Masslynx (µg ml^{-1})
D = any dilution factor used
W = sample equivalent weight (0.25 g for the quantities in the procedure above)

The sample equivalent weight (W) is calculated as follows:
W (g) = M/Vext
M = mass of maize sample extracted (5 g)
Vext = volume of the extraction solvent (20 mL)

Data Analysis

Combined analysis of variance (ANOVA) was performed on FER symptoms, *F. verticillioides* target DNA and fumonisin concentrations in maize grain using Proc GLM of SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). The GEI demonstrated by ANOVA was further analysed by additive main effects and multiplicative interaction (AMMI) using Genstat v16 (Payne *et al*., 2013). The AMMI model partitioned GEI into the first and second interaction principal components axes (IPCA) and residual to demonstrate stability of the genotypes across locations The distance from a genotype's coordinate point to the origin in a two-dimensional scatter diagram determines the AMMI stability value (ASV) (Purchase *et al*., 2000). Genotypes with the lowest ASV values exhibited the shortest projection from the biplot origin, and were considered the most stable.

The genotypic means relative to the principal components was graphically represented in GGE biplots generated in GenStat v16. The first principle component (PC1), located on the X-axis, indicated the level of resistance where inbred lines with higher PC1 values (positive or negative) were considered low risk to FER, *F. verticillioides* colonisation and fumonisin accumulation. The second principle component (PC2), located on the Y-axis,

represented performance stability of genotypes where PC2 values near zero demonstrated greater adaptability of genotypes to different environments (Yan and Kang, 2003). The GGE biplot graphically represented the genotype main effect and GEI (Yan *et al*., 2000; Yan, 2001). The method is based on evaluating genotypes, firstly by considering only the effects of the genotype and G x E as significant, while simultaneously taking these variables (G + GE) into consideration. Secondly, it enabled the evaluation and representation of genotypes in different environments using the biplot technique (Gabriel, 1971).

Mega-environments were determined on the "ideal genotype for a particular environment" or the "which-won-where" model. This approach determined the best performing genotypes which shared the same environments across years. The estimation of resistance and stability of genotypes to FER, *F. verticillioides* colonisation and fumonisin accumulation was evaluated by an average environment coordination (AEC) method (Yan *et al*., 2000; Yan, 2001). It uses the average principal components in all environments, represented as a circle, with a line drawn through the average environment and the biplot origin called the average environment axis (AEA). The perpendicular line passing through the AEA and the biplot origin, called the average ordinate environment (AOE), divided genotypes exhibiting above-average (to the right of the AOE) and below-average resistance (to the left of the AOE). Genotypes were projected on the AEA and ranked on resistance (low FER, *F. verticillioides* target DNA, fumonisins) with increased resistance in the direction of the arrow. The arrow pointed to a greater genotype main effect while the AEC ordinate and either direction away from the biplot origin indicated greater GEI effect and reduced stability. Concentric circles helped visualise the distance between each genotype and the ideal genotype, with a genotype being more desirable if it was located close to the ideal genotype. GGE biplots were constructed with genotype-focus and symmetrical scaling.

RESULTS

Fusarium ear rot severity

Inbred line response across seasons and locations: Disease development in the 2010/11 and 2011/12 seasons did not differ significantly ($P \leq 0.05$), with mean FER development on maize ears of 10.5 and 10.1%, respectively (data not shown). FER development was significantly ($P \leq 0.05$) influenced by the trial location, inbred line evaluated and the interactions between the two (Table 2A and 3). Genotype x environment interaction explained 42.8% of the total variation (G + E + GEI), whereas genotype and environment explained 34.3 and 21.9% of the variation observed, respectively. The first and second IPCA were both significant ($P = 0.00$), while PCA1 of the interaction accounted for 28.2% of the total GEI variation for FER severity, and PCA2 explained an additional 23.7% of the

observed variation (Table 3). The two PCA's, thus, accounted for 51.9% of the variability in FER disease expression.

In the 2010/11 season, the mean FER severity observed at Makhatini (3.0%) and Vaalharts (6.3%) differed significantly from each other and was significantly lower than at Cedara (16.1%), Potchefstroom (14.6%) and Buffelsvallei (12.5%) (Table 4). In the following season (2011/12), disease severity at Cedara (3.3%) was significantly lower than at Vaalharts (13.9%), Makhatini (12.3%) and Potchefstroom (10.8%). Symptom development at Vaalharts, Makhatini and Potchefstroom, however, did not differ significantly from one another. Maize inbred lines differed significantly in FER development at all field locations during the 2010/11 and 2011/12 season (Table 4). Overall, inbred line US 2540W (1.7%) expressed the least FER which did not differ significantly from VO 617y-2 (4.0%), CML 390 (5.2%), RO 544W (5.4%) and CB-248 (5.9%). Inbred lines I-37 (21.2%), I-35 (19.4%), I-9 (16.6%) and R119W (16.6%) were significantly more diseased than the other inbred lines evaluated, but did not differ significantly from each other (Table 4).

Stability of inbred line performance: Inbred lines that were most stable across locations (indicated by ASV) were I-34 (0.20), CB-248 (0.23) and RO 549W (0.37), while R119W (1.67), K64R-2 (1.10) and R2565y (1.09) exhibited inconsistent responses to FER severity across locations (Table 5). Assessment of FER severity by GGE biplot analyses revealed that the first two principal components accounted for 54.2 (PC1) and 15.4% (PC2) of the GGE variation, respectively (Fig. 2). The inbred lines US 2540W (#9), VO 617y-2 (#3), R2565y (#7), I16 (#5), I-37 (#16), I-35 (#10) and R119W (#19) delimited the polygon formed when these vertex genotypes were connected by straight lines (Fig. 2A). They, thus, differed most in their response to FER across environments.

Considerable crossover interactions were obtained, as demonstrated by the test localities falling into different sectors. The maize inbred line US 2540W (#9) exhibited the lowest FER symptoms at Buffelsvallei and Potchefstroom in the 2010/11 season, and in Vaalharts and Cedara in the 2011/12 season, to form the first mega-environment (Fig. 2A). Inbred lines RO 424W (#12) and CML 390 (#22) were also resistant to FER at the same locations and in the same years, as they fell into the same sector as US 2540W (#9). Inbred line VO 617y-2 (#3) exhibited the lowest FER symptoms at three localities (Potchefstroom 2011/12, Vaalharts 2010/11 and Cedara 2010/11), to form the second mega-environment. Inbred lines CB-248 (#21) and RO 549W (#8) were also resistant to FER, since they grouped close to the biplot origin, and were stable in their response due to low PC1 and PC2 scores, respectively (Fig. 2A; Table 3). Inbred line R119W (#19) was most susceptible to FER, but unstable in disease expression across locations (Fig. 2; Table 7).

Inbred line CML 390 (#22; 0.47) demonstrated the lowest FER severity and was the most stable in its response to the disease across localities (Fig. 2B). It was, therefore,

considered not to be affected by GEI. Other resistant genotypes that were also stable in their response to FER included inbred lines VO 617y-2 (#3; 0.89), US 2540W (#9; 0.55), RO 549W (#8; 0.37), RO 424W (#12; 0.49) and RO 544W (#13; 0.41). Furthermore, resistant inbred lines I-B (#17; 0.61) and CB-248 (#21; 0.23) were stable across localities while inbred lines CML 444 (#18; 0.86) and CB-222 (#20; 0.73) differed in their resistant response at various localities. Inbred lines I-34 (#6; 0.2), I-37 (#16; 0.42) and I-9 (#14; 0.45), however, were highly susceptible to FER, but stable in their response across environments (Fig. 2B; Table 5).

***Fusarium verticillioides* colonisation**

Inbred line response across locations: Contamination of maize grain with *F. verticillioides* was influenced by the production season, trial location, inbred line and the interactions amongst these factors (Table 2B and 3). The GEI effect contributed the most to the total variation (G + E + GEI) and explained 50.1% of the variation observed, while the genotype and environment explained 28.5 and 21.4% of the variation observed, respectively. The first and second IPCA were both significant (P = 0.00) and accounted for 35.9 and 21.7%, respectively, of the total variation, together explaining 57.6% of the GEI variability in fungal colonisation (Table 3).

The mean quantity of *F. verticillioides* target DNA in grain collected from five localities in the 2010/11 and four localities in the 2011/12 seasons differed significantly ($P \leq 0.05$), measuring 0.147 ŋg μl^{-1} and 0.118 ŋg μl^{-1}, respectively (data not shown). In 2010/11 the mean *F. verticillioides* target DNA quantified in grain collected in Makhatini (0.09 ŋg μl^{-1}) was significantly less than in those collected in Vaalharts (0.14 ŋg μl^{-1}), Cedara (0.15 ŋg μl^{-1}) and Buffelsvlei (0.24 ŋg μl^{-1}), but not from that in Potchefstroom (0.11 ŋg μl^{-1}) (Table 6). For the 2011/12 season, however, *F. verticillioides* target DNA quantified in grain collected in Cedara (0.06 ŋg μl^{-1}) was significantly less than those collected in Potchefstroom (0.12 ŋg μl^{-1}) and Makhatini (0.23 ŋg μl^{-1}), but not significantly less than in Vaalharts (0.07 ŋg μl^{-1}). The mean fungal target DNA quantified in grain from Makhatini, however, was significantly more than that quantified at Potchefstroom (Table 6).

Significant differences in fungal content were determined in grain collected from maize inbred lines from all locations during the 2010/11 and 2011/12 seasons (Table 6). Significantly less *F. verticillioides* target DNA was found in inbred lines CML 390 (0.04 ŋg μl^{-1}), RO 424W (0.06 ŋg μl^{-1}), CML 444 (0.07 ŋg μl^{-1}), US 2540W (0.07 ŋg μl^{-1}), VO 617y-2 (0.09 ŋg μl^{-1}), CB-222 (0.90 ŋg μl^{-1}), RO 544W (0.10 ŋg μl^{-1}), CB-248 (0.10 ŋg μl^{-1}) and I-37 (0.10 ŋg μl^{-1}) than in R119W (0.22 ŋg μl^{-1}), I-9 (0.25 ŋg μl^{-1}), I-35 (0.27 ŋg μl^{-1}) and I-16 (0.28 ŋg μl^{-1}), which contained the most fungal target DNA (Table 6).

Stability of inbred line performance: Inbred lines K64R-2 (0.16), I-B (0.21) and CB-222 (0.41) were the most stable in their response to *F. verticillioides* colonisation across environments, while VO 617y-2 (2.05), CML 444 (-0.64) and CML 390 (-0.08) were most inconsistent in their response (Table 5). GGE biplot analyses explained 67.9% (PC1 53.8% and PC2 14.1%) of the total GGE variation (Fig. 3). The inbred lines VO 617y-2 (#3), CML 390 (#22), CML 444 (#18), R119W (#19), I-35 (#10), I-16 (#5) and RO 549W (#8) differed the most in their response to *F. verticillioides* colonisation and outlined the polygon (Fig. 3A). Significant crossover interaction was indicated by test localities falling into different sectors. VO 617y-2 (#3) accumulated the least fungal target DNA at Buffelsvallei during the 2010/11 season and at Potchefstroom, Vaalharts and Cedara during the 2011/12 season, thereby forming a mega-environment (Fig. 3A). Inbred lines RO 424W (#12) and US 2540W (#9) also resisted fungal colonisation at the same localities and grouped with inbred line VO 617y-2 (#3) into the same sector. Inbred line CML 390 (#22) was resistant to fungal colonisation in multiple environments over the two seasons (Makhatini in 2010/11 and 2011/12, and Vaalharts, Cedara and Potchefstroom in 2010/11), to form a second mega-environment (Fig. 3A).

Inbred lines CB-222 (#20; 0.40), RO 424W (#12; 0.80) and US 2540W (#9; 1.19) were resistant to *F. verticillioides* colonisation, but were unstable in their response to fungal colonisation across locations. Inbred lines R119W (#19; 0.64), I-16 (#5; 0.64), I-35 (#10; 1.23) and I-9 (#14; 0.84) were consistently susceptibility to *F. verticillioides* colonisation across all environments (Fig. 3A; Table 7). Overall, inbred line CML 390 (#22) was the most resistant to *F. verticillioides* colonisation and most stable in its response across locations (Fig. 3B). Inbred lines US 2540W (#9) and RO 424W (#12) were also resistant to *F. verticillioides* colonisation based on their proximity to the AEC. Inbred lines R119W (#19), I-35 (#10), I-34 (#6), I-16 (#5) and I-9 (#14) were most susceptible to *F. verticillioides* colonisation across locations (Fig. 3B).

Fumonisin accumulation

Inbred line response across locations: The planting season, trial location, inbred line and interactions between these factors all significantly ($P \leq 0.05$) influenced the total fumonisins accumulated in maize grain (Table 2C and 3). The environmental effect was responsible for the largest variation in fumonisin accumulation, and explained 43.7% of the total variation (G + E + GEI). The GEI and genotype contributed 32.0 and 10.9% of the total variation, respectively. The IPCA1 and IPCA 2 were both significant and explained 42.7 and 21.2% of the total variability in fumonisin accumulation, respectively (Table 3).

The mean total fumonisin accumulated in 2010/11 differed significantly ($P \leq 0.05$) from that accumulated in the 2011/12 seasons, totaling 10.8 and 7.2 mg kg^{-1}, respectively (data not shown). The mean fumonisin contamination of grain collected at Makhatini (4.6 mg kg^{-1}) in 2010/11 was significantly less than that collected at Cedara (16.8 mg kg^{-1}), which was

significantly more than at any other locality (Table 7). In the 2011/12 season, grain from Makhatini (18.6 mg kg^{-1}) accumulated significantly more total fumonisins than all the other locations, which did not differ significantly from each other (Table 7).

The fumonisin contamination of grain collected from the maize inbred lines differed at the individual locations during both growing seasons (Table 7). When data from inbred lines at the different locations were combined, inbred lines CML 390 (4.1 mg kg^{-1}), RO 424W (4.2 mg kg^{-1}), CB-222 (4.9 mg kg^{-1}), US 2540W (5.6 mg kg^{-1}), CB-248 (5.6 mg kg^{-1}), CML 444 (6.1 mg kg^{-1}), K64R-2 (7.1 mg kg^{-1}) and R2565y (7.4 mg kg^{-1}) accumulated the least fumonisins and did not differ significantly from each other. Inbred lines R119W (16.9 mg kg^{-1}), I-16 (15.0 mg kg^{-1}), I-9 (15.3 mg kg^{-1}), I-35 (13.6 mg kg^{-1}) and R0549W (12.9 mg kg^{-1}) were most contaminated with fumonisins and did not differ significantly from one another (Table 7).

Stability of inbred line performance: The stability of inbred line to fumonisin accumulation across environments, as determined by ASV, ranged from 0.55 to 2.17. Inbred lines R2565y (0.55), I-B (0.55) and I-16 (0.58) were the most stable, while RO 549W (2.17), VO 617y-2 (1.66) and I-34 (1.51) were the most unstable (Table 5).

The PC1 (44.3%) and PC2 (19.0%) accounted for a total of 63.3% of the GGE variation (Fig. 4). Inbred line CML 390 (#22) exhibited the lowest fumonisin accumulation in all localities across 2 years, creating a single mega-environment. The locations had crossover interactions, as demonstrated by their division into different sectors of the polygon view (Fig. 4A). Inbred lines RO 424W (#12) and US 2540W (#9) were also resistant to fumonisin accumulation across localities and occupied the same sector as inbred line CML 390 (#22). Furthermore, inbred lines RO 424W (0.70) and US 2540W (0.76) were stable in their response to fumonisin accumulation (Table 5). Inbred lines I-16 (#5; 0.58), I-9 (#14; 1.11), R119W (#19; 0.61), I-35 (#10; 1.44) and I-34 (#6; 1.51) were most susceptible to fumonisin accumulation and differed in the stability of their response (Fig. 4A; Table 3). Inbred line CML 390 (#22) was most resistant and most stable in its response to fumonisin accumulation across localities, while lines R119W (#19), I-35 (#10), I-34 (#6), I-16 (#5), and I-9 (#14) were most susceptible to fumonisin accumulation (Fig. 4B).

Correlations between FER severity, *Fusarium verticillioides* colonisation and fumonisin contamination

Significant correlations between FER severity, fungal colonisation and fumonisin accumulation were observed at all localities in both seasons (Table 8). In 2010/11, moderate correlations between FER and *F. verticillioides* colonisation was determined at Buffelsvallei (R = 0.57) and Potchefstroom (R = 0.57) while a poor, though significant correlation was determined at Cedara (R = 0.32). Insignificant correlations were observed at Makhatini (R = 0.24; P = 0.0750) and Vaalharts (R = 0.19; P = 0.1733). The relationship between FER and

total fumonisin content was insignificant at four of the five localities planted in 2010/11 with a moderate correlation between these parameters only determined at Potchefstroom (R = 0.56). Conversely, moderate to strong positive correlations were determined between *F. verticillioides* colonisation and total fumonisin content of grain from maize inbred lines at all localities during 2010/11 (Table 8).

In 2011/12 poor, though significant correlations were determined between FER and fungal content in grain at Cedara (R = 0.41) and Vaalharts (R = 0.36) while insignificant correlations were established for Makhatini (R = 0.12; P = 0.3754) and Potchefstroom (R = 0.26; P = 0.0596) (Table 8). The correlation between FER and fumonisin content at Makhatini (R = 0.29), Potchefstroom (R = 0.40) and Vaalharts (R = 0.36) were significantly poor and insignificant at Cedara (R = 0.25; P = 0.0735). Moderate to strong positive correlations were again determined between *F. verticillioides* colonisation and total fumonisin content of grain from maize inbred lines at all localities during the 2011/12 season (Table 8).

Over the two seasons the correlation between FER and *F. verticillioides* colonisation at Cedara (R = 0.47), Makhatini (R = 0.30) and Potchefstroom (R = 0.39) was significantly moderate to poor and insignificant at Vaalharts (R = 0.09; P = 0.3356). The association between FER and fumonisin content in maize grain was significantly moderate at Cedara (R = 0.40), Makhatini (R = 0.45) and Potchefstroom (R = 0.46), and insignificant at Vaalharts (R = 0.10; P = 0.3076). Strong positive correlations between fungal and fumonisin content of maize grain were observed at all localities evaluated over the two seasons (Table 8). In the overall study, FER and *F. verticillioides* colonisation (R = 0.36) as well as FER and fumonisin content (R = 0.31) correlated poorly while *F. verticillioides* colonisation and total fumonisins correlated well (R = 0.64).

Climatic conditions

Significant differences in maximum temperature, relative humidity and maximum rainfall was observed between the localities in both seasons (Table 9). In 2010/11 and 2011/12, Makhatini was significantly hotter than all other localities while Cedara was significantly cooler in both seasons. Overall, Makhatini was significantly hotter when compared to the other localities while the maximum temperature at Potchefstroom and Buffelsvallei did not differ significantly over the 2 years. Cedara was significantly cooler over the 2-year evaluation period. Conversely, Cedara had the highest relative humidity during both seasons when compared to all other localities, while Vaalharts had the lowest percentage relative humidity during the 2011/12 season. Overall, the percentage relative humidity at Buffelsvallei and Cedara did not differ significantly and was significantly higher than at all other localities. Significantly more rain was recorded at Buffelsvallei (2010/11) than at all other localities for both seasons, while significantly less rainfall was recorded at Vaalharts during the 2010/11

and 2011/12 season. The rainfall at Vaalharts (2010/11) did not differ significantly from that at Makhatini (2010/11), Cedara (2011/12) and Makhatini (2011/12) (Table 9).

DISCUSSION

Maize cultivars grown in South Africa are highly susceptible to FER and fumonisin contamination, with fumonisin levels often exceeding the tolerable limits enforced in Europe and the USA (Rheeder *et al*., 1990; Janse van Rensburg *et al*., 2015). Sources of resistance to *F. verticillioides* and fumonisin accumulation are, therefore, urgently needed to develop resistant cultivars for use in commercial and subsistence farming systems. In this study, maize inbred lines CML 390, US 2540W and RO 424W were identified as resistant to FER, *F. verticillioides* colonisation and fumonisin accumulation across different environments. Resistance to FER and fumonisin accumulation by CML 390 has previously been reported (Small *et al*., 2012). The resistant inbred lines identified in this study could potentially be used in plant improvement programmes to reduce the risk of fumonisin contamination in the country.

Inbred lines VO 617y-2 and RO 549W, although resistant to FER and fungal colonisation across environments, were not resistant to fumonisin accumulation. Inbred line CB-222 was resistant to FER and fumonisin accumulation, but susceptible to fungal colonisation. These results suggest that the genetic potential for resistance to FER (disease expression), fungal colonisation and fumonisin accumulation may exist independently of each other, and is determined by the maize genotype.

A significant GEI was observed for all the inbred lines evaluated in this study. The GEI contributed more to the variation in FER severity and *F. verticillioides* colonisation than to fumonisin production, while the environment contributed largely to the variation in fumonisin accumulation. These results highlight the ability to screen for resistance to FER and *F. verticillioides* colonisation in environments that are conducive to the proliferation of the fungus, while the suitability of an environment for evaluating resistance to fumonisin accumulation should initially be established. Differential response of inbred lines to FER and fumonisin accumulation due to GEI has previously been documented (Clements *et al*., 2004; Afolabi *et al*., 2007; Small *et al*., 2012). Additionally, Cao *et al*. (2014) showed that fumonisin contamination was predominantly affected by the prevailing environmental conditions. This was also observed by de la Campa *et al*. (2005) when modelling the effects of environment, insect damage and *Bt* genotypes on fumonisin accumulation. The environment's substantial influence on fumonisin accumulation accentuates the value of evaluating resistance to fumonisin accumulation in target environments.

Significant differences in climatic data between localities contributed to the GEI effect observed in this study. Furthermore, maximum temperature, rainfall and relative humidity

provided conditions for the growth of *F. verticillioides* and the deposition of fumonisins. The highest FER severity and fumonisin contamination in 2010/11 season at Cedara coincided with significantly cooler temperatures (<25^{o}C) but the highest percentage relative humidity. High FER severity, fungal and fumonisin contamination at Makhatini during 2011/12 occurred under hot (<30^{o}C) and humid conditions (<90% RH). These results suggest that an environment with high relative humidity and maximum temperature that ensures the growth of *F. verticillioides* provides an ideal setting to evaluate resistance to fumonisin accumulation. Humidity clearly plays an important role in fumonisin contamination. High levels of fumonisins have been reported under hot and dry weather conditions (Shelby *et al*., 1994), whereas high levels of fumonisins was associated with high relative humidity in Argentina (Hennigen *et al*., 2000).

While inbred lines CML 390, US 2540W and RO 424W were resistant to *F. verticillioides* infection and fumonisin accumulation across localities and seasons, others such as RO 549W, CML 444 and CB-222 were resistant only in specific environments. It thus appears as if resistant genotypes can have either a broad or specific adaptability to environments. Estimating stability parameters for genotype response to *F. verticillioides* is thus essential to determine their value across environments. Consequently, hybrids developed from resistant inbred lines could be used to resist *F. verticillioides* and fumonisin accumulation in specific agro-ecological zones.

Significant correlations between FER severity, *F. verticillioides* colonisation and fumonisin accumulation were found in this study. A significant though moderate relationship between FER and fungal target DNA and FER and fumonisin content, demonstrates the limitation of solely employing visual assessment to identify sources of resistance. Although the selection of visibly healthy genotypes should, consequently, also exhibit low *F. verticillioides* colonisation and fumonisin accumulation, numerous fumonisin-resistant genotypes may be discarded based solely on FER severity. Moderate to high heritability and strong genetic correlation between FER and fumonisin resistance in two maize populations determined that visual selection for reduced FER should result in reduced fumonisins (Robertson-Hoyt *et al*., 2006). Later generations of inbreds selected for reduced FER, however, warranted evaluation for reduced fumonisin accumulation.

The relationship between FER severity, *F. verticillioides* colonisation and fumonisin accumulation was reduced by inbred lines that exhibited low FER severity but with high fungal colonisation and fumonisin content. Inbred lines with low FER severity, high levels of fungal colonisation and low fumonisin concentrations were also observed in this study. The occurrence of similar discrepancies highlights the necessity for fumonisin analysis when evaluating maize for resistance (Afolabi *et al*., 2007; Small *et al*., 2012). Asymptomatic kernels with high levels of infection and low fumonisin concentrations have been reported (Desjardins *et al*., 1998; Bush *et al*., 2004). Factors which affect kernel infection might

operate independently from those that affect fumonisin production, thereby affecting the relationship between these two variables (Clements and White, 2004). These results reflect the need for quantifying fumonisins when identifying sources of resistance to *F. verticillioides* and fumonisin accumulation.

The significantly strong correlation between fungal colonisation and fumonisin accumulation illustrates the potential of these parameters, in addition to visual disease severity, to more accurately identify sources of resistance. The quantification of *F. verticillioides* target DNA in maize grain will provide a better indication of the potential risk for fumonisin contamination (Janse van Rensburg *et al*., 2015). The associated cost and expertise required to quantify fungal target DNA or fumonisins may not be practical. This should be weighed against the gains in identifying resistant genotypes to *F. verticillioides* and fumonisin accumulation more accurately, as breeding strategies based on disease severity alone have not demonstrated effective management of fumonisin contamination.

The use of AMMI and GGE biplot analysis to determine the stability of disease resistance through multi-location trials has been described before (Sharma *et al*., 1987; Rubiales *et al*., 2012; Sibiya *et al*., 2012, 2013; Pande *et al*., 2013). These analyses are employed for the first time in this study to determine and characterise resistance to FER and fumonisin accumulation in maize inbred lines. Functionalities of GGE biplot analysis enabled the comparison of genotypes in all environments and the determination of best performing genotypes in specific environments. The division of trial localities among different sectors of the polygon view of the GGE biplot further supports the importance of evaluating potential breeding material for resistance to *F. verticillioides* and fumonisin accumulation over several diverse environments.

A 2-year study across five field localities in South Africa was undertaken to investigate the response of maize inbred lines to FER severity, *F. verticillioides* colonisation and fumonisin accumulation. The identification of inbred lines resistant to FER and fumonisin accumulation across environments is important, as these lines can be used by seed companies as well-adapted breeding materials. The four inbred lines that displayed stable resistance to *F. verticillioides* and fumonisin accumulation could be used in future to develop maize hybrids resistant to FER and fumonisin accumulation. These lines, furthermore, provide the opportunity to expand the knowledge base on the genetic processes governing resistance to *F. verticillioides* and fumonisin accumulation in maize.

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Table 1. Maize inbred lines evaluated for resistance to Fusarium ear rot, *Fusarium verticillioides* colonisation and fumonisin accumulation in South Africa during the 2010/11 and 2011/12 maize growing seasons.

Line Number	Inbred line	Origin[1]	FER/fumonisin[2]	Kernel Colour	Protein Content
3	**VO 617y-2**	ARC-GCI- South Africa	Resistant[2]	Yellow	High lysine
4	**K64R-22**[3]	ARC-GCI- South Africa	Intermediate[2]	White	Normal
5	**I-16**	ARC-GCI- South Africa	Unknown	White	Unknown
6	**I-34**	ARC-GCI- South Africa	Unknown	White	Unknown
7	**R2565y**	ARC-GCI- South Africa	Susceptible[2]	Yellow	Normal
8	**RO 549W**	ARC-GCI- South Africa	Resistant[2]	White	High lysine
9	**US 2540**	ARC-GCI- South Africa	Resistant[2]	White	Normal
10	**I-35**	ARC-GCI- South Africa	Unknown	White	Unknown
12	**RO 424W**	ARC-GCI- South Africa	Intermediate[2]	White	High lysine
13	**RO 544W**	ARC-GCI- South Africa	Susceptible[2]	White	High lysine
14	**I-9**	ARC-GCI- South Africa	Unknown	White	Unknown
16	**I-37**	ARC-GCI- South Africa	Unknown	White	Unknown
17	**I-B**	ARC-GCI- South Africa	Intermediate[2]	White	Normal
18	**CML 444**[3]	CIMMYT-Zimbabwe	Resistant[2]	White	Normal
19	**R119W**	ARC-GCI- South Africa	Resistant[2]	White	Normal
20	**CB-222**	ARC-GCI- South Africa	Unknown	White	Unknown
21	**CB-248**	ARC-GCI- South Africa	Unknown	White	Unknown
22	**CML 390**	CIMMYT-Zimbabwe	Resistant[2]	White	Normal

[1]Agricultural Research Council – Grain Crops Institute (ARC-GCI), CIMMYT – International Maize and Wheat Improvement Center.
[2]According to (Small *et al*., 2012; Mouton, 2014).
[3]Inbred line characterised as drought resistant (Cairns *et al*., 2013)

Table 2. Analysis of variance of **A)** Fusarium ear rot, **B)** *Fusarium verticillioides* colonisation and **C)** total fumonisin content determined across all years, all locations and for all inbred lines evaluated.

A)

Source of variation	Df	SS	MS	F-value	Pr>F
Year	1	0.71	0.706	1.34	0.2479
Location	4	10.57	2.643	5.02	0.0006
Year x Location	3	95.23	31.742	60.29	<0.0001
Year x Location (Rep)	18	83.02	4.612	8.76	<0.0001
Inbred line	17	172.07	10.122	19.23	<0.0001
Year x Inbred line	17	23.81	1.400	2.66	0.0005
Location x Inbred line	68	109.71	1.613	3.06	<0.0001
Year x Location x Inbred line	51	74.88	1.468	2.79	<0.0001

B)

Source of variation	Df	SS	MS	F-value	Pr>F
Year	1	4.46	4.458	9.34	0.0024
Location	4	31.62	7.904	16.57	<0.0001
Year x Location	3	64.06	21.355	44.76	<0.0001
Year x Location (Rep)	18	12.39	0.688	1.44	0.1102
Inbred line	17	133.34	7.844	16.44	<0.0001
Year x Inbred line	17	27.63	1.625	3.41	<0.0001
Location x Inbred line	68	140.86	2.071	4.34	<0.0001
Year x Location x Inbred line	51	65.65	1.287	2.7	<0.0001

C)

Source of variation	Df	SS	MS	F-value	Pr>F
Year	1	28.10	28.102	120.7	<0.0001
Location	4	23.97	5.990	25.74	<0.0001
Year x Location	3	109.95	26.650	157.41	<0.0001
Year x Location (Rep)	18	4.40	0.244	1.05	0.4037
Inbred line	17	53.27	3.134	13.46	<0.0001
Year x Inbred line	17	16.94	0.996	4.28	<0.0001
Location x Inbred line	68	97.75	1.438	6.17	<0.0001
Year x Location x Inbred line	51	41.02	0.804	3.45	<0.0001

Df – degrees of freedom
SS – Sum of squares
MS – Mean square
Data converted to natural log of observed or measured values

Table 3. Additive main effects and multiplicative interaction analysis of variance of Fusarium ear rot (FER) disease severity, *Fusarium verticillioides* colonisation and total fumonisin content of maize inbred lines evaluated in South Africa.

		FER severity				*Fusarium verticillioides* colonisation				Total fumonisins			
Source of variation	***Df***	***SS***	***MS***	***Pr>F***	***Total variation***	***SS***	***MS***	***Pr>F***	***Total variation***	***SS***	***MS***	***Pr>F***	***Total variation***
Treatment	161	487	3.03	0.0		467.6	2.9	0		371	2.3	0.0	
Environment	8	106.5	13.31	0.0	21.9	100.1	12.52	0	21.4	162	20.25	0.0	43.7
Block	18	83	4.61	0.0	-	12.4	0.69	0.11	-	4.4	0.24	0.4	-
Genotype	17	172.1	10.12	0.0	34.3	133.3	7.84	0	28.5	53.3	3.13	0.0	10.9
Interaction	136	208.4	1.53	0.0	42.8	234.1	1.72	0	50.1	155.7	1.15	0.0	32.0
IPCA1	24	58.7	2.45	0.0	28.2	84.1	3.5	0	35.9	66.5	2.77	0.0	42.7
IPCA2	22	49.4	2.24	0.0	23.7	50.8	2.31	0	21.7	33	1.5	0.0	21.2
Residual	90	100.3	1.12	0.0	-	99.3	1.1	0	-	56.3	0.63	0.0	-
Error	306	161.1	0.53	*		146	0.48	*		71.2	0.23	*	
Total	485	731.1	1.51	*		626	1.29	*		446.7	0.92	*	

Natural log transformation used in analyses
IPCA – Interaction Principle Component Axis scores

Table 4. Fusarium ear rot symptoms of maize inbred lines planted at multiple field locations in South Africa during the 2010/11 and 2011/12 maize-growing seasons.

		Fusarium ear rot severity (%)[1,2,3]										
Line #	***Inbred name***	***Buffelsvallei***		***Cedara***		***Makhatini***		***Potchefstroom***		***Vaalharts***		***Combined***
		2010/2011	**2011/2012**	**2010/2011**	**2011/2012**	**2010/2011**	**2011/2012**	**2010/2011**	**2011/2012**	**2010/2011**	**2011/2012**	***mean***
3	**VO 617y-2**	1.5 d	-	0.9 h	3.6 a-c	3.0 bc	9.8 bc	2.8 de	0.0 d	1.4 fg	12.6 c-e	4.0 fg
4	**K64R-22**	23.5 bc	-	33.5 a-c	10.1 a	0.5 bc	7.8 bc	30.7 ab	10.3 b-d	12.9 ab	5.0 ed	14.9 b-d
5	**I-16**	21.0 bc	-	37.0 ab	6.9 ab	4.0 bc	14.7 bc	23.8 a-c	1.2 d	6.9 b-g	18.7 a-d	14.9 b-d
6	**I-34**	21.8 bc	-	8.9 e-h	5.1 a-c	3.8 bc	9.7 bc	22.6 a-c	18.1 b-d	10.4 a-c	23.4 a-c	13.7 cd
7	**R2565y**	13.8 cd	-	5.2 f-h	1.0 bc	12.5 a	6.5 bc	23.3 a-c	3.0 cd	0.3 g	31.8 ab	10.8 de
8	**RO 549W**	1.3 d	-	15.9 d-h	0.9 bc	0.5 bc	12 bc	15.7 b-e	0.0 d	7.5 b-f	2.5 e	6.3 e-g
9	**US 2540**	0.5 d	-	3.1 gh	0.0 c	2.5 bc	3.4 c	0.9 e	1.6 d	2.2 e-g	1.3 e	1.7 g
10	**I-35**	40.5 a	-	27.4 a-d	3.0 bc	1.8 bc	10.3 bc	28.8 a-c	40.8 a	8.9 b-e	12.6 c-e	19.4 ab
12	**RO 424W**	2.9 d	-	2.0 h	2.3 bc	4.8 bc	14 bc	5.5 de	3.3 cd	4.6 c-g	21.8 a-d	6.8 ef
13	**RO 544W**	3.6 d	-	22.2 b-e	6.9 ab	2.1 bc	2.2 c	3.9 de	0.0 d	2.7 d-g	4.8 ed	5.4 fg
14	**I-9**	29.3 ab	-	31.3 a-d	5.9 a-c	5.1 b	16.3 a-c	28.3 a-c	11.4 b-d	9.7 a-d	11.7 c-e	16.6 a-c
16	**I-37**	34.5 ab	-	18.8 c-g	5.6 a-c	2.9 bc	11.6 bc	39.0 a	26.2 ab	16.6 a	35.8 a	21.2 a
17	**I-B**	5.5 d	-	7.7 e-h	1.7 bc	5.0 b	10.4 bc	4.4 de	4.0 cd	1.6 fg	25.8 a-c	7.3 ef
18	**CML 444**	5.6 d	-	19.5 c-f	2.3 bc	1.1 bc	33.2 a	0.1 e	4.5 cd	2.1 e-g	1.2 e	7.7 ef
19	**R119W**	0.0 d	-	38.6 a	1.8 bc	1.8 bc	22.2 ab	18.3 b-d	41.7 a	16.2 a	8.8 c-e	16.6 a-c
20	**CB-222**	5.5 d	-	3.6 gh	0.1 c	0.3 c	10.8 bc	2.7 de	20.8 bc	6.5 b-g	15.6 b-e	7.3 fg
21	**CB-248**	11.6 cd	-	4.1 f-h	1.2 bc	2.4 bc	6.1 bc	12 c-e	8.1 b-d	3.5 c-g	3.9 ed	5.9 fg
22	**CML 390**	3.1 d	-	10.0 e-h	0.5 bc	0.5 bc	19.6 a-c	0.6 e	0.0 d	0.2 g	12.6 c-e	5.2 fg
	Mean	**12.5 b**		**16.1 a**	**3.3 b**	**3.0 d**	**12.3 a**	**14.6 ab**	**10.8 a**	**6.3 c**	**13.9 a**	***10.3***
	LSD	***14.5***	***-***	***15.9***	***6.6***	***4.8***	***18.0***	***17.1***	***18.7***	***7.2***	***18.0***	***4.7***

[1]The percentage of maize ears covered with visual symptoms of Fusarium ear rot.
[2]The mean of disease severity for three field plots.
[3]Means followed by the same alphabetical letter in each column are not significantly different according to the Student's t-test of least significant differences ($P \geq 0.05$).

Table 5. Additive main effects and multiplicative interaction analysis stability values (ASVs) and interaction principle component axes (IPCA) values for Fusarium ear rot, *Fusarium verticillioides* colonisation and total fumonisin content of maize inbred lines evaluated in South Africa.

		FER severity[1]			***Fusarium verticillioides* colonisation[2]**			**Total fumonisins[3]**		
Line number	*Inbred line*	*ASV*	*IPCA1*	*IPCA2*	*ASV*	*IPCA1*	*IPCA2*	*ASV*	*IPCA1*	*IPCA2*
3	**VO617y-2**	0.8906	0.19	-0.86	2.0463	-1.21	-0.46	1.66	-0.82	0.13
4	**K64R-2**	1.10	0.31	1.03	0.1632	0.05	0.14	1.244	-0.62	0.07
5	**I 16**	0.7849	0.64	0.21	0.6408	0.28	-0.44	0.58	-0.01	0.58
6	**I 34**	0.20	0.06	0.19	1.20	0.73	0.06	1.508	0.73	0.31
7	**R2565y**	1.087	0.77	-0.59	0.6127	0.36	-0.13	0.548	0.27	-0.09
8	**RO 549W**	0.3663	0.20	0.28	1.1255	-0.24	-1.05	2.166	-1.07	-0.07
9	**US 2540W**	0.5467	-0.38	-0.31	1.1924	-0.71	-0.21	0.76	-0.36	0.21
10	**I 35**	0.7398	-0.24	0.68	1.2281	0.74	0.11	1.439	0.71	0.02
12	**RO 424W**	0.4911	0.03	-0.49	0.80	-0.47	-0.19	0.70	-0.34	0.11
13	**RO 544W**	0.4078	0.30	0.2	0.8266	0.38	0.53	0.789	-0.37	0.26
14	**I 9**	0.4512	0.08	0.44	0.8406	0.43	-0.45	1.109	0.45	0.63
16	**I 37**	0.4233	0.04	0.42	0.8548	0.48	-0.31	0.965	0.42	0.48
17	**I-B**	0.6128	0.38	-0.41	0.2088	-0.12	-0.07	0.549	0.21	-0.34
18	**CLM 444**	0.8592	-0.66	-0.35	1.5698	-0.64	1.16	1.30	0.12	-1.27
19	**R119W**	1.6687	-1.40	0.06	0.6442	0.21	0.54	0.605	0.26	-0.30
20	**CB 222**	0.7324	-0.60	-0.15	0.40	0.17	0.29	1.092	0.50	-0.42
21	**CB 248**	0.2289	0.17	0.10	0.5359	0.32	0.07	0.609	0.30	0.00
22	**CML 390**	0.4696	0.11	-0.45	1.3504	-0.08	0.41	0.844	-0.39	-0.31
	Mean	***0.6***			***0.78***			***0.9***		

[1]The percentage of maize ears covered with visual symptoms of Fusarium ear rot
[2]The absolute concentrations of *F. verticillioides* target DNA
[3]Total fumonisin content as the sum of FB_1, FB_2 and FB_3 for three field plots for the 2010/2011 and 2011/2012 seasons combined.

Table 6. *Fusarium verticillioides* colonisation of maize inbred lines planted at multiple field locations in South Africa during the 2010/11 and 2011/12 maize-growing seasons.

		***Fusarium verticillioides* target DNA (ng uL^{-1})[1,2,3]**										
Line #	***Inbred name***	***Buffelsvallei***		***Cedara***		***Makhatini***		***Potchefstroom***		***Vaalharts***		***Combined mean***
		2010/2011	**2011/2012**	**2010/2011**	**2011/2012**	**2010/2011**	**2011/2012**	**2010/2011**	**2011/2012**	**2010/2011**	**2011/2012**	
3	**VO 617y-2**	0.02 d	-	0.33 a	0.02 c	0.13 a	0.18 c-g	0.03 d	0.01 d	0.05 d-e	0.05 b-d	0.09 d-f
4	**K64R-22**	0.15 cd	-	0.18 a-f	0.88 a-c	0.09 ab	0.17 d-g	0.05 d	0.25 a-c	0.11 c-f	0.01 d	0.12 c-e
5	**I-16**	0.48 a-c	-	0.29 ab	0.15 a	0.09 ab	0.56 a	0.30 ab	0.12 b-d	0.37 a	0.13 ab	0.28 a
6	**I-34**	0.20 cd	-	0.04 f	0.07 a-c	0.11 a	0.25 cd	0.16 b-d	0.31 ab	0.23 bc	0.04 b-d	0.16 bc
7	**R2565y**	0.16 cd	-	0.15 b-f	0.02 c	0.08 ab	0.14 d-g	0.13 b-d	0.20 b-d	0.10 c-f	0.15 a	0.13 c-e
8	**RO 549W**	0.02 d	-	0.28 a-c	0.02 c	0.08 ab	0.08 g	0.14 b-d	0.03 cd	0.31 ab	0.03 cd	0.11 c-e
9	**US 2540**	0.01 d	-	0.06 f	0.03 c	0.10 ab	0.33 bc	0.02 d	0.02 cd	0.05 d-f	0.02 d	0.07 ef
10	**I-35**	0.87 a	-	0.10 d-f	0.05 a-c	0.09 ab	0.13 d-g	0.28 a-c	0.43 a	0.40 a	0.09 a-d	0.27 a
12	**RO 424W**	0.04 cd	-	0.13 c-f	0.05 bc	0.06 ab	0.12 d-g	0.05 d	0.02 cd	0.03 ef	0.04 b-d	0.06 ef
13	**RO 544W**	0.26 b-d	-	0.12 c-f	0.0 4c	0.06 ab	0.10 e-g	0.05 d	0.16 b-d	0.03 ef	0.08 a-d	0.99 c-f
14	**I-9**	0.67 ab	-	0.23 a-e	0.08 a-c	0.11 ab	0.47 ab	0.41 a	0.11 b-d	0.17 cd	0.02 d	0.25 a
16	**I-37**	0.21 cd	-	0.08 ef	0.03 c	0.02 b	0.23 c-f	0.13 b-d	0.04 cd	0.12 c-f	0.06 b-d	0.10 c-f
17	**I-B**	0.07 cd	-	0.12 c-f	0.04 bc	0.11 a	0.56 a	0.07 d	0.09 b-d	0.16 cd	0.13 ab	0.15 b-d
18	**CML 444**	0.04 cd	-	0.08 ef	0.13 ab	0.06 ab	0.13 d-g	0.00 d	0.07 cd	0.05 d-f	0.04 b-d	0.07 ef
19	**R119W**	0.81 a	-	0.27 a-d	0.08 a-c	0.11 a	0.25 c-e	0.06 d	0.13 b-d	0.12 c-f	0.12 a-c	0.22 ab
20	**CB-222**	0.16 cd	-	0.10 ef	0.02 c	0.06 ab	0.11 d-g	0.02 d	0.09 b-d	0.18 b-d	0.08 a-d	0.09 c-f
21	**CB-248**	0.19 cd	-	0.06 f	0.03 c	0.09 ab	0.16 d-g	0.09 cd	0.06 cd	0.07 d-f	0.16 a	0.10 c-f
22	**CML 390**	0.03 cd	-	0.09 ef	0.03 c	0.09 ab	0.09 fg	0.01 d	0.01 d	0.01 f	0.03 cd	0.04 f
	Mean	***0.24 a***	***-***	***0.15 b***	***0.06 c***	***0.09 c***	***0.23 a***	***0.11 bc***	***0.12 b***	***0.14 bc***	***0.07 c***	***0.18***
	LSD	***0.45***	***-***	***0.17***	***0.10***	***0.09***	***0.15***	***0.21***	***0.23***	***0.14***	***0.09***	***0.07***

[1]The means of absolute concentrations of *F. verticillioides* DNA for three field plots, with each field plot represented by the average of two technical replications for qPCR.
[2]The mean of fungal target DNA for three field plots.
[3]Means followed by the same alphabetical letter in each column are not significantly different according to the Student's t-test of least significant differences ($P \geq 0.05$).

Table 7. Total fumonisin content of maize inbred lines planted at multiple field locations in South Africa during the 2010/11 and 2011/12 maize-growing seasons.

		Total fumonisins (mg kg^{-1})[1,2,3]										
Line #	***Inbred name***	***Buffelsvallei***		***Cedara***		***Makhatini***		***Potchefstroom***		***Vaalharts***		***Combined mean***
		2010/2011	**2011/2012**	**2010/2011**	**2011/2012**	**2010/2011**	**2011/2012**	**2010/2011**	**2011/2012**	**2010/2011**	**2011/2012**	
3	**VO 617y-2**	1.9 g	-	35.6 b	1.7 d	5.1 a-c	25.7 a-f	5.7 cd	0.0 e	5.2 d	4.4 ab	9.5 d-f
4	**K64R-22**	4.8 gf	-	22.8 cd	3.2 b-d	5.0 a-c	15.2 d-g	4.3 cd	0.0 e	7.7 cd	0.9 e	7.1 e-i
5	**I-16**	18.6 cd	-	18.6 de	5.5 bc	0.7 c	31.1 a-c	25.7 a-c	2.1 de	27.7 a	5.2 a	15.0 ab
6	**I-34**	18.6 cd	-	3.8 g	1.7 d	5.8 a-c	27.9 a-d	15.2 a-d	7.7 bc	10.4 cd	3.1 a-e	10.5 c-e
7	**R2565y**	4.8 gf	-	13.3 d-g	0.9 d	3.9 a-c	15.9 c-g	7.5 cd	7.9 bc	9.6 cd	2.7 b-e	7.4 e-i
8	**RO 549W**	1.5 g	-	74.4 a	4.1 b-d	4.7 a-c	10.9 gf	5.9 cd	0.0 e	13.6 b-d	1.0 de	12.9 b-d
9	**US 2540**	1.7 g	-	7 gf	0.6 d	4.0 a-c	26.8 a-e	4.1 cd	0.0 e	5.0 d	1.3 de	5.6 g-i
10	**I-35**	13.4 de	-	8.7 e-g	1.9 cd	5.8 a-c	10.9 gf	37.0 a	17.6 e	23.9 ab	3.1 a-e	13.6 a-c
12	**RO 424W**	2.7 g	-	9.1 e-g	2.1 cd	2.9 bc	10.8 gf	4.0 cd	0.0 e	4.2 d	1.8 c-e	4.2 i
13	**RO 544W**	24.5 c	-	20.1 d	2.7 b-d	5.1 a-c	17.2 b-g	3.8 cd	0.0 e	4.1 d	2.0 c-e	8.8 e-g
14	**I-9**	33.3 b	-	15.2 d-f	2.7 b-d	9.1 a	25.9 a-f	33.8 ab	6.1 b-d	9.4 cd	1.8 c-e	15.3 ab
16	**I-37**	12.2 de	-	6.8 gf	3.4 b-d	2.0 c	13.8 d-g	24.2 a-d	3.8 c-e	7.5 cd	2.8 b-e	8.5 e-h
17	**I-B**	5.5 e-g	-	9.8 e-g	2.4 cd	8.1 ab	33.9 a	4.9 cd	7.3 b-d	17.1 a-c	3.7 a-c	10.3 c-e
18	**CML 444**	5.1 gf	-	8.2 gf	10.9 a	3.4 a-c	11.1 e-g	0.2 d	6.7 b-d	6.1 d	2.9 a-e	6.1 f-i
19	**R119W**	47.2 a	-	30.8 bc	6.4 b	6 a-c	33 ab	5.0 cd	9.9 b	10.6 cd	3.2 a-d	16.9 a
20	**CB-222**	8.8 e-g	-	5.2 gf	2 cd	1.8 c	6.8 g	2.7 cd	6.4 b-d	8.3 cd	2.1 b-e	4.9 hi
21	**CB-248**	6.5 e-g	-	4.8 g	3 b-d	5.2 a-c	8.2 g	10.8 b-d	3.9 c-e	5.0 d	3.2 a-d	5.6 g-i
22	**CML 390**	3.3 g	-	8.3 gf	3.9 b-d	3.6 a-c	10.4 gf	1.7 cd	0.0 e	3.9 d	1.3 de	4.1 i
	Mean	***11.90 b***	**-**	***16.8 a***	***3.3 b***	***4.6 c***	***18.6 a***	***10.9 b***	***4.4 b***	***10.0 b***	***2.6 b***	***9.23***
	LSD	***7.91***	**-**	***10.2***	***3.7***	***6.0***	***15.8***	***24.4***	***5.4***	***10.7***	***2.3***	***3.74***

[1]Total fumonisin content is the sum of FB_1, FB_2 and FB_3.
[2]The mean fumonisin concentration for three field plots.
[3]Means followed by the same alphabetical letter in each column are not significantly different according to the Student's t-test of least significant differences ($P \geq 0.05$).

Table 8. Correlations between Fusarium ear rot severity, *Fusarium verticillioides* colonisation and total fumonisin content at multiple field locations in South Africa during the 2010/11 and 2011/12 maize-growing seasons.

	2010/11 season			2011/12 season			Combined across locality		
Locality	***FER/ Fv***	***FER/ FUMS***	***Fv/ FUMS***	***FER/ Fv***	***FER/ FUMS***	***Fv/ FUMS***	***FER/ Fv***	***FER/ FUMS***	***Fv/ FUMS***
Buffelsvallei	0.57	0.17* *P* = 0.2209	0.59	N/A	N/A	N/A	0.57	0.17* *P* = 0.2209	0.59
Cedara	0.32	0.22* *P* = 0.1154	0.64	0.41	0.25* *P* = 0.0735	0.67	0.47	0.40	0.70
Makhatini	0.24* *P* = 0.0750	0.19* *P* = 0.1606	0.57	0.12* *P* = 0.3754	0.29	0.72	0.30	0.45	0.79
Potchefstroom	0.57	0.56	0.92	0.26* *P* = 0.0596	0.40	0.69	0.39	0.46	0.72
Vaalharts	0.19* *P* = 0.1733	0.06* *P* = 0.6693	0.89	0.36	0.36	0.58	0.09* *P* = 0.3356	0.10* *P* = 0.3076	0.84
Combined	**0.43**	**0.36**	**0.59**	**0.24**	**0.25**	**0.73**	**0.36**	**0.31**	**0.64**

* Not significant (*P*>0.05)
FER – Fusarium ear rot severity (%)
Fv - *Fusarium verticillioides* target DNA concentration (ng uL^{-1})
FUMS - Total fumonisin content (FB_1+FB_2+FB_3) (mg kg^{-1})

Table 9. Maximum temperature, relative humidity and maximum rainfall at multiple field locations in South Africa during the 2010/11 and 2011/12 maize-growing seasons.

	Maximum Temperature (°C)			Relative Humidity (%)			Maximum Rainfall (mm)		
Locality	***2010/11***	***2011/12***	***Combined***	***2010/11***	***2011/12***	***Combined***	***2010/11***	***2011/12***	***Combined***
Buffelsvallei	25.7 d	-	**25.7 c**	93.5 ab	-	**93.5 a**	130.5 a	-	**130.5 a**
Cedara	24.1 f	24.6 ef	**24.4 d**	95.9 a	95.7 a	**95.8 a**	87.8 bc	55.8 c-e	**71.8 bc**
Makhatini	30.9 a	31.0 a	**30.9 a**	89.8 cd	91.6 bc	**90.7 b**	55.5 c-e	36.7 de	**46.1 cd**
Potchefstroom	25.2 de	26.7 c	**25.9 c**	89.9 cd	86.7 d	**88.3 bc**	115.0 ab	68.7 cd	**91.8 b**
Vaalharts	28.4 b	30.3 a	**29.4 b**	90.6 bc	82.9 e	**86.7 c**	21.1 e	41.0 de	**31.0 d**

Means followed by the same alphabetical letter in each column as well as across the 2010/11 and 2011/12 seasons are not significantly different according to the Student's t-test of least significant differences ($P \geq 0.05$).

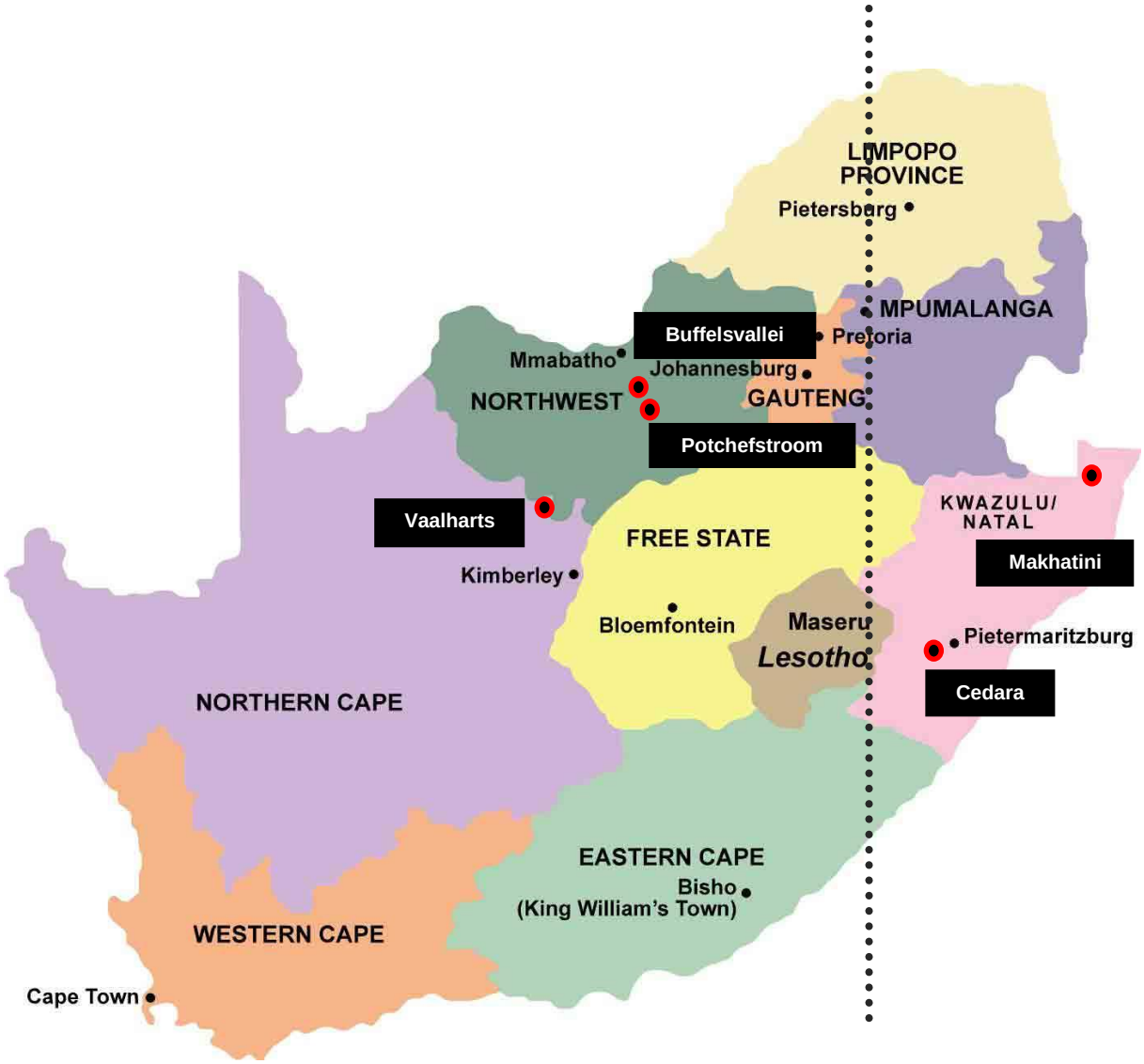

Figure 1. Locations where maize inbred lines were evaluated for resistance against *Fusarium verticillioides* included Buffelsvallei and Potchefstroom (North-West province), Vaalharts (Northern Cape province) and Cedara and Makhatini (KwaZulu-Natal province) in South Africa.

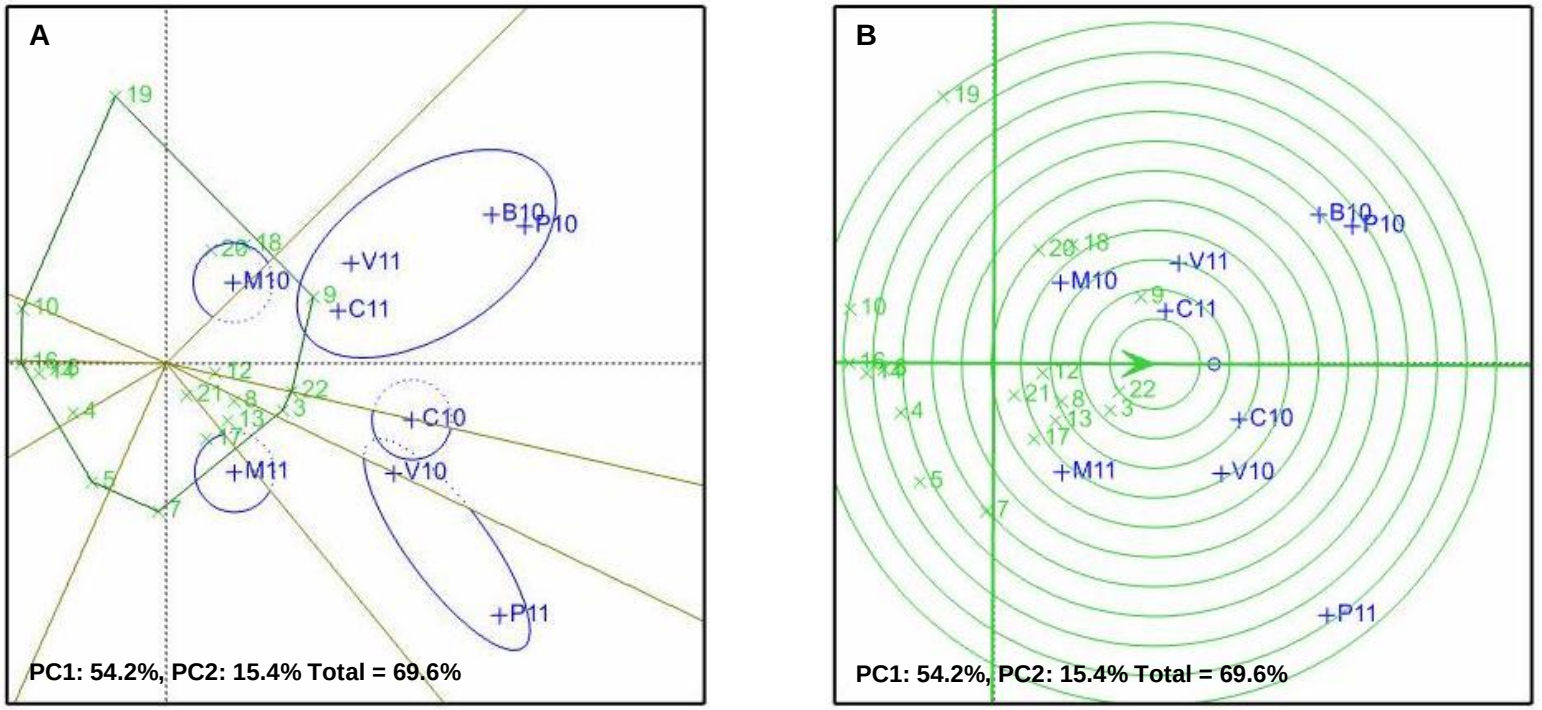

Figure 2. Genotype main effect and genotype by environment interaction biplot based on environment-focused scaling for **A)** the comparison of genotypes and **B)** the polygon view exhibiting mega-environments for Fusarium ear rot severity in 18 maize inbred lines tested during the 2010/11 and 2011/12 seasons. Green and blue numbers represent genotypes and environments, respectively, with localities represented as: Buffelsvallei 2010/11 [B10], Cedara 2010/11; 2011/12 [C10; C11], Makhatini 2010/11; 2011/12 [M10; M11], Potchefstroom 2010/11; 2011/12 [P10; P11], Vaalharts 2010/11; 2011/12 [V10; V11].

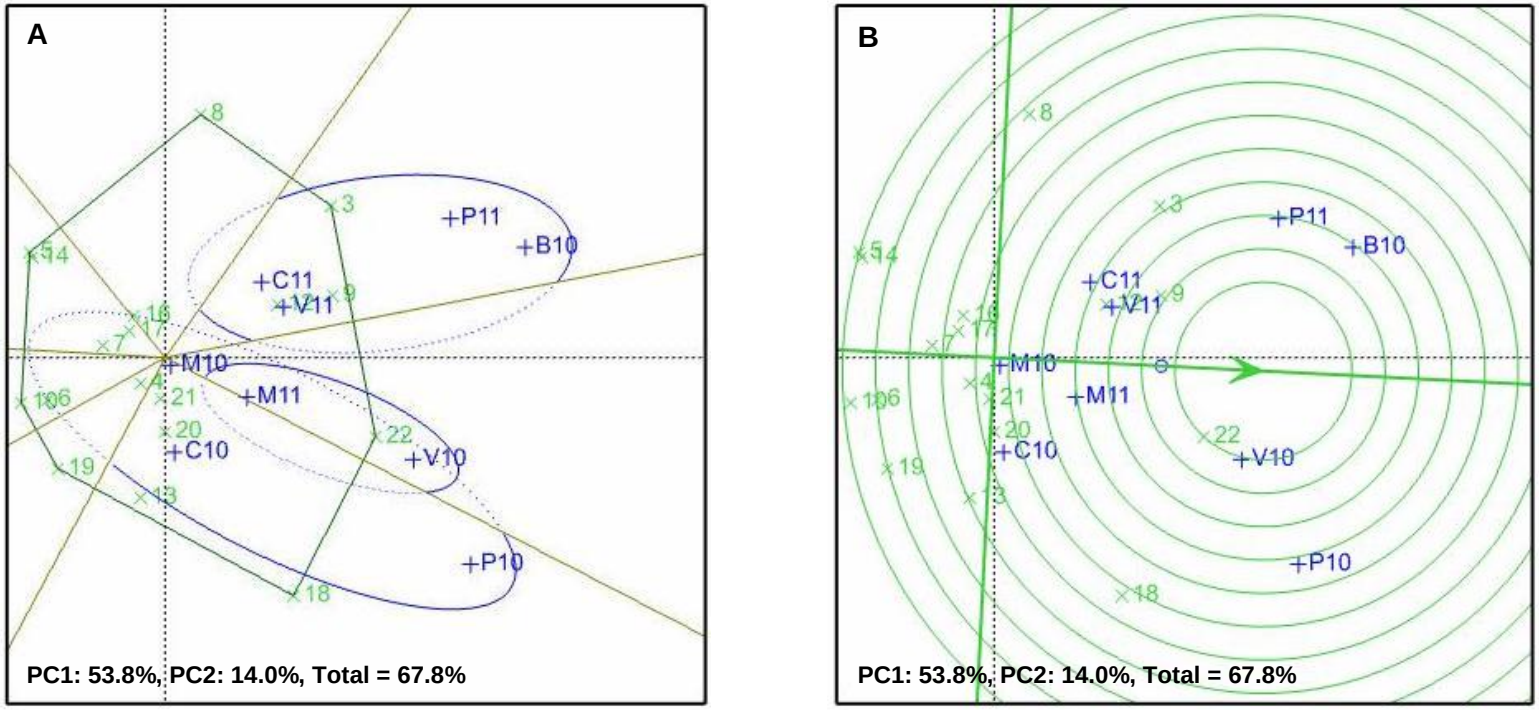

Figure 3. Genotype main effect and genotype by environment interaction biplot based on environment-focused scaling for **A)** the comparison of genotypes and **B)** the polygon view exhibiting mega-environments for *F. verticillioides* colonisation in 18 maize inbred lines tested during the 2010/11 and 2011/12 seasons. Green and blue numbers represent genotypes and environments, respectively, with localities represented as: Buffelsvallei 2010/11 [B10], Cedara 2010/11; 2011/12 [C10; C11], Makhatini 2010/11; 2011/12 [M10; M11], Potchefstroom 2010/11; 2011/12 [P10; P11], Vaalharts 2010/11; 2011/12 [V10; V11].

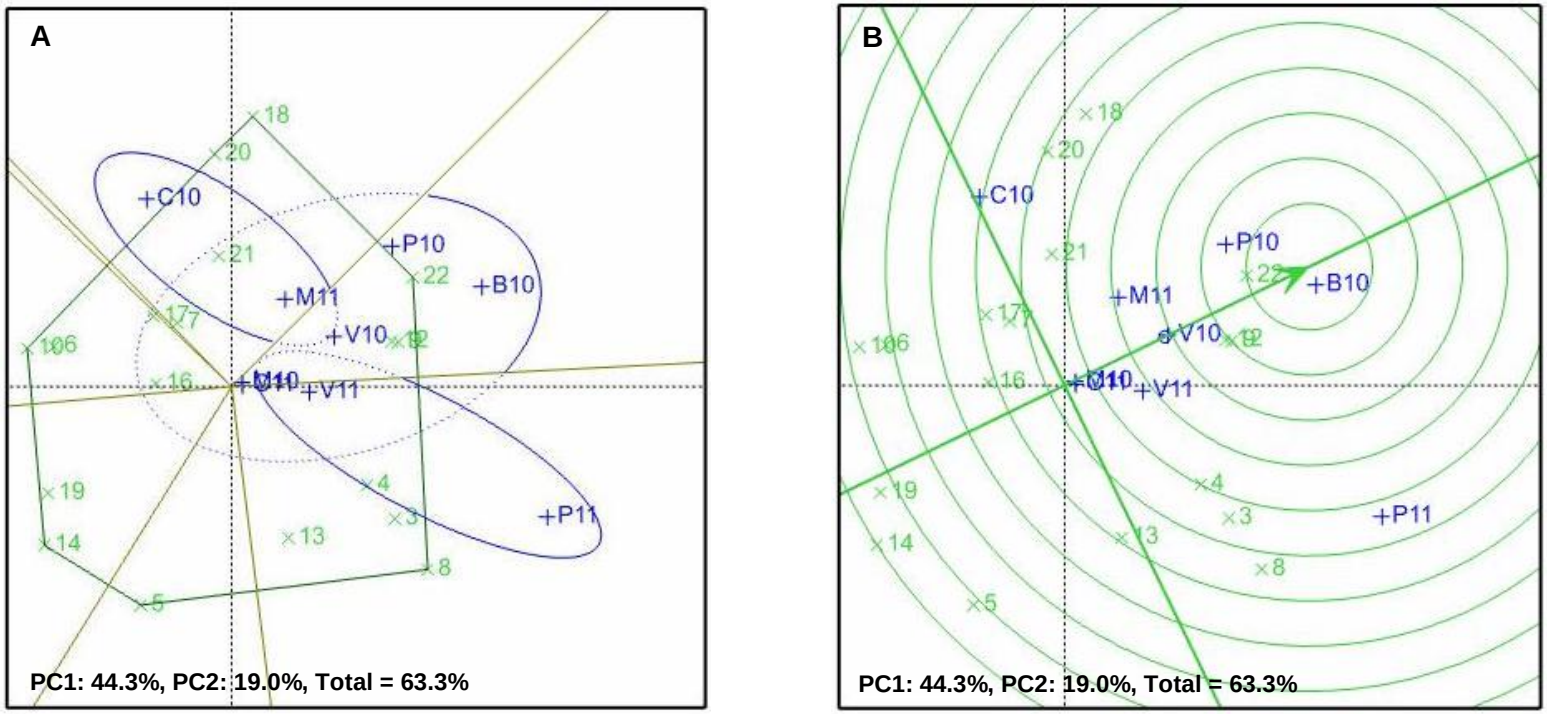

Figure 4. Genotype main effect and genotype by environment interaction biplot based on environment-focused scaling for **A)** the comparison of genotypes and **B)** the polygon view exhibiting mega-environments for total fumonisin accumulation in 18 maize inbred lines tested during the 2010/11 and 2011/12 seasons. Green and blue numbers represent genotypes and environments, respectively, with localities represented as: Buffelsvallei 2010/11 [B10], Cedara 2010/11; 2011/12 [C10; C11], Makhatini 2010/11; 2011/12 [M10; M11], Potchefstroom 2010/11; 2011/12 [P10; P11], Vaalharts 2010/11; 2011/12 [V10; V11].

CHAPTER 3

Evaluating maize inbred lines, resistant to Aspergillus ear rot and aflatoxin accumulation, for resistance to *Fusarium verticillioides* and fumonisin contamination in South Africa and Kenya

ABSTRACT

The fungal plant pathogens *Fusarium verticillioides* and *Aspergillus flavus* cause Fusarium ear rot (FER) and Aspergillus ear rot (AER) of maize, respectively. Both pathogens are of concern to producers as they reduce grain yield and quality. Moreover, *F. verticillioides* and *A. flavus* contaminate maize grain with the mycotoxins fumonisins and aflatoxins, respectively. Both toxins have been associated with mycotoxicosis in humans and animals. The occurrence of common resistance mechanisms to FER and AER makes the identification of sources of resistance to one pathogen an opportune means to reduce disease and mycotoxin contamination by the other pathogen. In this study, 10 Kenyan inbred lines resistant to AER and aflatoxin accumulation were evaluated for resistance to FER, *F. verticillioides* colonisation and fumonisin accumulation. Nine South African inbred lines resistant to FER and fumonisin accumulation, as well as two susceptible lines, were included in the trials. Field trials were conducted at three localities in South Africa and two localities in Kenya over one season. FER severity was determined by visual assessment, while *F. verticillioides* colonisation and fumonisin content were quantified by real-time PCR and liquid chromatography tandem mass spectrometry, respectively. Significant genotype x environment interactions were found at each location ($P \leq 0.05$). The Kenyan inbred line CML 495 was most resistant to FER and *F. verticillioides* colonisation, and accumulated the lowest concentration of fumonisins across localities. It was, however, not significantly more resistant than the Kenyan inbred lines CML 264 and CKL05015, and the South African line RO 549W, which also exhibited low FER severity (≤5%), fungal target DNA (≤0.05 ng μL^{-1}) and fumonisin levels (≤3.0 mg kg^{-1}). Inbred lines resistant to AER and aflatoxin accumulation appeared to be good sources of resistance to *F. verticillioides* and fumonisin contamination.

INTRODUCTION

Fusarium ear rot (FER) and Aspergillus ear rot (AER) are important diseases of maize in Africa as they reduce both grain yield and quality. The diseases are caused by the fungal pathogens *Fusarium verticillioides* (Sacc.) Nirenberg and *Aspergillus flavus* Link:Fr., respectively. *Fusarium verticillioides* and *A. flavus* produce toxic secondary metabolites in maize grain, called mycotoxins, which pose a serious threat to human and animal health. Fumonisins, the mycotoxin produced by *F. verticillioides*, have been associated with oesophageal cancer of human adults in South Africa, Italy, Iran and China (Franceshi *et al*., 1990; Rheeder *et al*., 1992; Shephard *et al*., 2000; Li *et al*., 2001) and neural tube defects in infants (Hendricks, 1999; Missmer *et al*., 2006). Aflatoxins, produced by *A. flavus*, are potent carcinogens and have resulted in severe cases of aflatoxicosis in Kenya which led to human fatalities (Williams *et al*., 2004). It also reduced the height of children in Benin and Togo (Gong *et al*., 2002) and suppressed the immune system of both humans and animals (Williams *et al*., 2004). Fumonisins and aflatoxins are poisonous to livestock too, with fumonisins causing equine leukoencephalomalacia (Kellerman *et al*., 1990) and porcine pulmonary oedema syndrome (Colvin and Harrison, 1992), while aflatoxins cause aflatoxicosis in cattle and sheep. Mycotoxin levels in food and feed are strictly regulated in the USA and European Union (FDA, 2000; European Commission, 2007). In South Africa, the maximum tolerable level for aflatoxin levels in foods is 10 ppb (Government Gazette, 2004), but no legislation exists for fumonisins in maize and maize-based products. Studies conducted in rural areas of South Africa indicated that fumonisin levels in maize grain often exceed tolerable limits enforced in Europe and USA (Shephard *et al*; 2007; Shephard, 2008; Ncube *et al.,* 2011).

Fumonisins and aflatoxins can co-occur on maize grain (Fandohan *et al*., 2005), but the nature of the interaction between their respective producers, *F. verticillioides* and *A. flavus*, is still unclear. It has been suggested that kernels infected by *F. verticillioides* may become resistant to subsequent infection by *A. flavus* (Wicklow *et al*., 1988). Marin *et al*. (1998) found that *F. verticillioides* outgrew *A. flavus* over a range of temperatures and reduced its growth *in vitro*. This supports the hypothesis of a competitive relationship between the two species, possibly for the same resources or niche on maize ears (Marin *et al*., 1998). Giorni *et al*. (2009), however, demonstrated that these fungi occupied different ecological niches based on carbon source utilization patterns *in vitro*. When plants were co-inoculated with *F. verticillioides* and *A. flavus*, their grain contained significantly less aflatoxins than grain from plants that were inoculated with *A. flavus* only (Zummo and Scott, 1992). Conversely, Abbas *et al*. (2006) reported that fumonisin and aflatoxin levels in hybrids naturally infected with *F. verticillioides* and *A. flavus* were positively correlated. These results

suggest that both species can grow on the same plant without necessarily competing for the same resources if host plants are highly susceptible.

The planting of resistant maize cultivars, as part of an integrated disease management strategy, is considered an efficient approach to reduce FER and AER and minimize the risk of mycotoxin accumulation in maize. The strong positive correlations between FER and AER severity, and also between AER and aflatoxin accumulation (Robertson-Hoyt *et al*., 2007), suggest that selecting maize germplasm for reduced FER should reduce AER and aflatoxin contamination. Quantitative trait loci (QTLs) associated with resistance to FER, AER, fumonisin and aflatoxin accumulation suggest that resistance to FER, AER and their associated mycotoxins may be genetically linked (Robertson-Hoyt *et al*., 2007). Xiang *et al*. (2010) further revealed that QTLs associated with resistance to FER, AER and Gibberella ear rot (GER), caused by *Fusarium graminearum* Schwabe, clustered in the same chromosome regions.

The first step in enhancing host-plant resistance in crops is to identify sources of resistance, preferably in locally-adapted breeding materials. Resistance, if durable under different growing conditions and in different maize production areas, can then be introduced into high-yielding and agronomically superior planting material that is susceptible to FER and fumonisin contamination. Plant resistance provides a number of advantages compared to other disease management options, such as ease of handling, affordability and being environmentally friendly. Maize inbred lines and hybrids have thus been evaluated worldwide for resistance to either FER/fumonisin contamination or AER/aflatoxin contamination (Clements and White, 2004; Robertson *et al*., 2006; Afolabi *et al*., 2007; Eller *et al*., 2010; Henry *et al*., 2009; Parsons and Munkvold, 2012), but seldom against both.

FER and fumonisin contamination is considered an important constraint to maize production in South Africa. AER and aflatoxin contamination, on the contrary, poses a limited threat in temperate maize production areas, and affects only subsistence farmers in northern KwaZulu-Natal where maize-growing conditions are subtropical. In eastern Africa, however, plant protection efforts are focused almost entirely on AER and aflatoxin contamination of maize, with little to no information available on other ear rot pathogens and their mycotoxins, including *F. verticillioides*. The objectives of this study, therefore, were to evaluate Kenyan inbred lines resistant to AER and aflatoxin accumulation for resistance to FER and fumonisin accumulation in South Africa and Kenya and to compare resistant Kenyan inbred lines to well-characterised FER/fumonisin-resistant South African inbred lines.

MATERIALS AND METHODS

Plant material evaluated

Ten Kenyan inbred lines, previously characterised as resistant to AER and aflatoxin accumulation (Dr. D. Makumbi, personal communication), and nine maize inbred lines from South Africa, previously characterised as resistant to FER and fumonisin accumulation (Small *et. al*. 2012; Mouton, 2014) were used in this study (Table 1). Additionally, two South African inbred lines susceptible to FER and fumonisin accumulation were included as susceptible checks. The South African inbred lines were provided by the Agricultural Research Council – Grain Crops Institute (ARC-GCI) in Potchefstroom, South Africa, while the Kenyan inbred lines were provided by the International Maize and Wheat Improvement Center (CIMMYT) in Nairobi, Kenya.

Field trials

Field trials in South Africa were conducted at Potchefstroom (grid ref.: 26˚73'S, 27˚07'E; altitude, 1 349 m) in the Northwest province and Vaalharts (grid ref.: 27˚95'S, 24˚83'E; altitude, 1 180 m) in the Northern Cape province during the 2012/13 maize growing season. A trial was also conducted at Makhatini (grid ref.: 22˚39'S, 32˚17'E; altitude, 77 m) in the KwaZulu-Natal province of South Africa during 2013, as this locality represents the most tropical maize-growing site in the country. In Kenya, screening trials were conducted in 2013 and performed at the Kenya Agricultural and Livestock Research Organisation (KALRO) stations of Katumani (grid ref.:1˚35'S, 37˚14'E; altitude1 600 m) and Kiboko (grid ref.:2˚15' S, 37˚75' E; altitude 975 m). These stations lie within the Machakos County in the semi-arid eastern Kenya, and has been shown to be an aflatoxin hot spot (Lewis *et al*., 2005).

Standard procedures to prepare fields were followed at all the trial locations. Maize kernels were manually planted (two seeds per hill) in double-row 10-m plots, with an intra-row spacing of 0.3 m and an inter-row spacing of 1 m. The trials were planted using a randomised complete block design and replicated three times, with experimental plots thinned to 33 plants per plot 3 weeks after emergence. The Potchefstroom and Makhatini trials were conducted under dryland conditions and supplemented with overhead irrigation when required, while the trial conducted at Vaalharts was flood irrigated weekly. The trials conducted at Katumani and Kiboko were drip irrigated.

Fungal isolates and production of inoculum

Country-specific, well-characterised toxigenic isolates were used to generate inoculum. Isolates obtained from the areas where the field trials were conducted were used wherever possible. Isolates of *F. verticillioides* used to inoculate South African trials consisted of GCI

315 and GCI 790, which were originally isolated from infected maize in Ndwedwe (KwaZulu-Natal) and Rushof (Northern Cape), respectively. The prolific fumonisin B_1 producer MRC 826 (Rheeder *et al*., 1992), isolated from infected maize in the Transkei region (South Africa), was also included to ensure a high level of pathogenicity and fumonisin production. Three Kenyan isolates of *F. verticillioides*; K5, K38 and K48; were used to inoculate the Kenyan field trials. These isolates were obtained from infected maize from the highly contaminated Nandi district.

Inoculum for disease screening trials was prepared by growing the *F. verticillioides* isolates in Armstrong liquid medium (Booth, 1971). After incubation at 27^{o}C for 3-4 days, the spores were separated from the mycelia by filtration through two layers of sterile cheesecloth. The spore suspension was then centrifuged at 3 500 G, the supernatant discarded and the conidial pellet washed three times using sterile distilled water (40 mL). After the final wash, the conidia were re-suspended in sterile, distilled water, and its concentration adjusted to 1 x 10^6 spores.mL^{-1} for each *F. verticillioides* isolate using a haemocytometer. Equal volumes of the three conidial suspensions per country were then combined to produce the final inoculum. The inoculum was kept at 4^{o}C prior to and during the inoculation process, and inoculum viability was confirmed by fungal growth on potato dextrose agar following field inoculations.

Inoculation and assessment of disease severity

Maize ears were inoculated at the R2 blister stage (Ritchie *et al*., 1993) according to the method described by Small *et al*. (2012). Once the inoculated ears reached physiological maturity and the grain dried to 12-18% moisture they were harvested, de-husked and FER symptoms visually assessed. Disease severity was determined by the percentage of each ear covered by visible symptoms of FER (Clements and White, 2004). Maize ears were then mechanically shelled and bulked per experimental plot. A 250-g kernel sample from each bulked plot was ground to produce fine maize flour using a Cyclotech sample mill (Foss Tecator, Hoganas, Sweden). The mill was thoroughly cleaned with high pressure air between each sample to avoid cross contamination. Flour samples were stored at -20°C until the extraction of fumonisins and genomic DNA was performed.

Quantification of *Fusarium verticillioides* in maize grain

DNA was isolated from both fungal cultures (0.02 g) and milled maize samples (2.0 g), according to the methods described by Boutigny *et al*. (2012). Mycelia of fungal cultures were first produced in 100 mL potato dextrose broth incubated at 25°C on a rotary shaker. After 2 weeks the mycelia was harvested by filtration through double layered, sterile cheesecloth, rinsed twice with autoclaved distilled water and freeze-died. The freeze-dried

mycelia of MRC 826, GCI 315, GCI 790, the Kenyan isolates (K5, K38, K48), as well as the maize samples were stored at -20°C until genomic DNA was extracted with the commercially available DNeasy® Plant Mini kit (QIAGEN), with some modifications. The initial DNA isolation step consisted of the CTAB/PVP lysis method, and an additional phenol extraction step was performed on fungal cultures prior to the use of the commercial kit (Boutigny *et al*., 2012). The quantity and purity of the DNA yield of each fungal and grain sample was determined with a NanoDrop ND-1000 spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). The DNA of grain samples were diluted to a concentration of 10 ng μL^{-1} with sterile distilled water and stored at 4°C until quantitative real-time polymerase chain reaction (qPCR) was performed. The DNA of *F. verticillioides* isolate MRC 826 was used as a positive control to produce fungal DNA standards for the absolute quantification of *F. verticillioides* in maize.

The fungal target DNA in maize samples was determined by qPCR according to Boutigny *et al*. (2012), using the species-specific primers Fver356 fwd / Fver412 rev (Nicolaisen *et al*., 2009). Initially, a standard curve was prepared by diluting *F. verticillioides* MRC 826 by 2^3, 2^5, 2^7, 2^9 and 2^{11}-fold in maize free of fungal DNA (10 ng µL). The standard curve was subjected to a strict set of criteria consisting of a slope of between -3.1 and -3.6 and linearity above 0.98. In addition, the ΔCt between extrapolated and measured Ct numbers on the undiluted sample had to be <0.5 (CRL-EM-01/ 08, 2008). Regression equations of standard curves created to detect *F. verticillioides* in the maize samples were highly significant (R^2 > 0.99). Following the generation of the standard curve, fungal/maize dilutions (2^3-, 2^5-, 2^7-fold dilutions) were aliquoted and stored at -20°C for inclusion in qPCR assays as positive controls. The sensitivity of the qPCR primer pair to detect and quantify the isolates used in the South African and Kenyan trials was also confirmed prior to the analyses of maize samples. DNA from maize samples were analysed in duplicate, and standard pathogen DNA (2^3-fold dilution of pathogen DNA in maize DNA free of *F. verticillioides* infection) were analysed in triplicate. All the assays contained a no template control (NTC). The presence of inhibitors in pathogen DNA, as well as intra- and inter-run variability of the qPCR assay for the quantification of *F. verticillioides* in infected maize samples, was previously validated by Boutigny *et al*. (2012).

Fumonisin quantification from maize grain

Fumonisins were extracted from milled maize samples as follows: Twenty mL methanol extraction buffer (MeOH/HPLC water; 70:30 v/v) was first added to each 5-g flour sample. The suspension was then shaken at 200 rpm in an incubator/shaker set at 25°C for 30 min. The samples were thereafter centrifuged at 500 G at 4°C for 10 min. A sterile syringe was used to remove ±2 mL of the clear extract, which was then filtered through a 0.20-µm

recombinant cellulose (RC) filter into a 2-mL Eppendorf tube. The samples were placed at 4^{o}C overnight, after which they were centrifuged for 10 min at 17.2 G before being transferred to LC-MS/MS glass vials. Samples for fumonisin analysis were diluted in a 1:1 ratio with HPLC-grade water and submitted to the Central Analytical Facility (CAF) at Stellenbosch University for the detection and quantification of FB_1, FB_2 and FB_3.

Fumonisin levels in maize samples were determined by the dilute-and-shoot method using liquid chromatographic tandem mass spectrometry (LC-MS/MS). The procedure was performed on a Quattro Micro triple quadrupole mass spectrometer from Waters/Micromass (Manchester, UK), equipped with an Alliance 2695 HPLC system (Waters) and Mass Lynx NT software 4.1 for data acquisition and processing. The electrospray ionization (ESI) source was used in the positive mode and the settings were optimized for the best sensitivity. Fumonisin standards (B_1 [10 mg], B_2 [10 mg], and B_3 [1 mg]), guaranteed 95% pure, were obtained from the Medical Research Council - Programme on Mycotoxins and Experimental Carcinogenesis (MRC-PROMEC), Tygerberg, South Africa. A dilution series that ranged between 0.05 and 20 mg kg^{-1} for FB_1 and FB_2, and between 0.005 and 2 mg kg^{-1} for FB_3 was analysed with the field trial samples. A calibration curve, performed with each set of samples analysed, consisted of six dilutions of a stock standard and a no-toxin control, represented by pure MeOH/water (70/30, v/v). Each standard and sample (5 µl) was injected into the LC-MS/MS system, and samples with results above of the calibration curve limit were appropriately diluted using HPLC-grade water and re-analysed. After adjustment for the volume of extract used in the purification procedure, the minimum limits of quantification for FB_1, FB_2 and FB_3 were 0.02 mg kg^{-1}, 0.002 mg kg^{-1} and 0.02 mg kg^{-1}, respectively.

Data Analysis

The data obtained from the visual assessment of FER symptoms, fumonisin accumulation and *F. verticillioides* target DNA determination was subjected to univariate Analysis of Variance (ANOVA), employing the Generalized linear model (GLM) procedure of SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). The student's t-test, which determines least significant difference (LSD) between treatment means, was calculated at a 95% confidence interval. Non-parametric Pearson correlation coefficients were determined to assess the relationships between FER symptoms, total fumonisin accumulated and fungal target DNA contamination using the CORR procedure in SAS employing the untransformed means of variables.

The data was furthermore subjected to additive main effects and multiplicative interaction (AMMI) analysis of variance (Gauch and Zobel, 1996) using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). The genotype by environment (G x E) interactions were partitioned amongst the first and second interaction principal

components axes (IPCA) and the residual. The first principle component (PC1), located on the X-axis, indicates the level of resistance where inbred lines with higher PC1 values (positive or negative) are considered low risk to FER disease severity, *F. verticillioides* colonisation or fumonisin accumulation. The second principle component (PC2), located on the Y-axis, represents performance stability of genotypes where PC2 values near zero demonstrate greater adaptability of genotypes to different environments (Yan and Kang, 2003).

The stability of the genotypes across locations was determined by the AMMI stability values (ASV) (Purchase *et al*., 2000), which is based on the first and second IPCA scores of the AMMI model for each genotype. The distance from a genotype's coordinate point to the origin in a two-dimensional scatter diagram determines the ASV value. Therefore, genotypes with the lowest ASV values exhibit the shortest projection from the biplot origin and are considered the most stable. Furthermore, the genotypic means relative to the principal components was graphically represented in GGE biplots. The GGE biplots were generated in GenStat 15th edition (Payne *et al*., 2013) by using the singular value decomposition (SVD) of the first two principle components (PC1 and PC2), according to Yan (2002). The GGE biplot graphically represented the genotype main effect and the G x E interaction (Yan, 2001; Yan *et al*., 2000). The method is based on evaluating genotypes, firstly by considering only the effects of the genotype and G x E as significant, while simultaneously taking these variables (G + GE) into consideration. Secondly, it enabled the evaluation and representation of genotypes in different environments using the biplot technique (Gabriel, 1971). Mega-environments were determined on the "ideal genotype for a particular environment" or the "which-won-where" approach. This approach determined the best performing genotypes shared in the same environments consistently across years.

The estimation of resistance and stability of genotypes to FER, *F. verticillioides* colonisation and fumonisin accumulation was evaluated by an average environment coordination (AEC) method (Yan and Hunt, 2000; Yan, 2001; Yan, 2002). It used the average principal components in all environments, represented as a circle, with a line drawn through the average environment and the biplot origin called the average environment axis (AEA). The perpendicular line passing through the AEA and the biplot origin, called the average ordinate environment (AEO), divided the genotypes into those exhibiting above-average resistance (to the right of the AEO) and those showing below-average resistance (to the left of the AEO). Genotypes were projected on the AEA and ranked on resistance (low FER, *F. verticillioides* target DNA, fumonisins), with increased resistance in the direction of the arrow. The arrow points to a greater genotype main effect, while the AEC ordinate, and either direction away from the biplot origin, indicates greater G x E interaction effect and reduced stability.

The PC1 and PC2 were also used to obtain the ideal test environment, characterised by large PC1 scores (more power to discriminate genotypes in terms of the genotypic main effect) and small (absolute) PC2 scores (more representative of the overall environments) (Yan and Rajcan, 2002). GGE biplots were constructed with genotype-focus and symmetrical scaling.

RESULTS

Fusarium ear rot severity

All the maize inbred lines evaluated in South Africa and Kenya developed FER symptoms. The mean level of disease severity at Makhatini (5.2%), Potchefstroom (8.0%), Vaalharts (7.8%) and Katumani (6.4%) was low and did not differ significantly from one another. At Kiboko (23.5%), however, the average FER development observed on maize inbred lines was significantly more severe (Table 2).

Under South African conditions, I137tnW (28.2; 23.3%) and R119W (26.8; 46.2%) developed significantly more FER symptoms at Makhatini and Potchefstroom, respectively, than the other inbred lines, whereas CML 444 (32.6%) was most susceptible to FER at Vaalharts (Table 2). Inbred lines VO 617y-2 (0.0%), CML 444 (0.2%), CML 264 (0.3%), CML 182 (0.5%), CML 495 (0.6%) and CKL05015 (1.1%) were most resistant to FER at Makhatini, and CKL05015 (0.4%), CML 390 (1.1%) and CML 264 (1.4%) at Potchefstroom. South African lines RO 549W (0.3%) and R2565y (0.5%), as well as Kenyan line CKL05015 (1.8%), were most resistant to FER at Vaalharts (Table 2). In Kenya, LaPosta (25.3%), CB 222 (23.8%) and US 2540W (14.0%) developed significantly more FER than the other inbred lines at Katumani, but not at Kiboko (Table 2). At the latter location, I137tnW (85.6%) and R119W (65.8%) were most susceptible to FER. The most resistant maize inbred lines at Katumani were CML 444 (0.0%), VO 617y-2 (0.2%), CML 495 (0.6%), CML 264 (0.8%) and CKL05022 (0.9%), and at Kiboko they were CKL05015 (0.6%), CML 247 (3.3%), CML 444 (3.3%) and CML 495 (3.6%). Over the five localities in the two countries R119W (20.8%) and I137tnW (19.0%) were most susceptible and inbred lines CML 495 (2.0%), CML 264 (2.5%) and VO 617y-2 (2.7%) most resistant to FER (Table 2).

FER symptoms were significantly ($P \leq 0.05$) influenced by the environment, genotype and the interactions between these factors (Table 5). The genotype x environment interactions (GEI) was responsible for the largest variation and explained 57.9% of the variation observed, followed by the genotype (25.0%) and environment (17.1%) effect. The first and second interaction principle component axis (IPCA) were both significant, with IPCA1 accounting for 25.4% of the total G x E variation and IPCA2 for 17.1% of the variation observed. In total, the two PCA's accounted for 42.5% of the variability in FER disease

symptom expression. The genotypes' response varied across locations, with ASV ranging from 0.35-1.69, with a mean of 0.82 (Table 6). Inbred lines CML 264 (0.35), MIRTC5 (0.45), CML 495 (0.46), P502c2 (0.48), CKL05003 (0.52), VO 617y-2 (0.53), LaPosta (0.54), US 2540W (0.61), CML 390 (0.68) and I137tnW (0.69) were the most stable in their response to FER across locations. Conversely, inbred lines CML 444 (1.69), CKL050019 (1.27), CKL050015 (1.12), CB 248 (1.08), RO 549W (1.05), CB 222 (1.04) and CKL05022 (1.02) were least stable in their expression of FER symptoms across locations (Table 6).

The genotype main effect and GEI was visualised by GGE biplot analysis (Fig. 1). In this study, the first two principal components accounted for 72.6% (PC1 = 47.2% and PC2 = 25.4%) of the total GGE variation for FER severity (Fig. 1). The genotype-ranking biplot revealed CML 495 (#12), CML 264 (#9) and VO 617y-e (#21) as the most resistant to FER, with CML 495 (#12) being the most stable (Fig. 1A). The genotype-comparison biplot supported this result, with CML 495 (#12) being found the ideal genotype with the lowest FER severity and greatest stability across localities (Fig. 1B). Inbred lines CKL05015 (#4), CML 264 (#9) and US 2540W (#20) were also identified as ideal genotypes, based on their proximity to CML 495. Lines I137tnW (#13) and R119W (#17) were the most susceptible genotypes (Fig. 1A and B). The polygon view, displaying the "which-won-where" pattern of the GGE biplot, revealed that inbred line CKL05015 (#4) was most resistant to FER at Makhatini, Potchefstroom and Kiboko, resulting in a mega-environment (Fig. 1C). Inbred lines US 2540W (#20) and CML 390 (#10) were also resistant to FER. Inbred CML 444 (#11) was most resistant to FER at Katumani, while CB 222 (#1) was most resistant in Vaalharts. Inbred lines CKL05015 (#4), CML 444 (#11), R119W (#17) and CB 222 (#1) served as vertex hybrids forming the polygon, and were the most affected by GEI due to their distance from the biplot origin. The high PC1 value and low PC2 score of the environment comparison biplot revealed Kiboko as the ideal test environment, followed by Potchefstroom and Makhatini (Fig. 1D).

Fusarium verticillioides colonisation

The mean quantity of *F. verticillioides* target DNA in maize inbred lines at Makhatini (0.029 ng uL^{-1}), Potchefstroom (0.033 ng uL) and Vaalharts (0.095 ng uL^{-1}) did not differ significantly from each other (Table 3). Inbred lines evaluated at these localities did, however, contain significantly less fungal target DNA than lines evaluated at Katumani (0.115 ng uL^{-1}) and Kiboko (0.529 ng uL^{-1}), with the exception of Vaalharts (Table 3).

At Makhatini, inbred lines CML 444 (0.007 ng uL^{-1}), CKL05019 (0.009 ng uL^{-1}), RO 549W (0.011 ng uL^{-1}), LaPosta (0.012 ng uL^{-1}) and CML 495 (0.013 ng uL^{-1}) were the least and inbred lines R119W (0.139 ng uL^{-1}) and CML 247 (0.063 ng uL^{-1}) the most contaminated with *F. verticillioides* (Table 3). At Potchefstroom, inbred lines CML 495 (0.003 ng uL^{-1}) and

CML 390 (0.005 ng uL^{-1}) accumulated the least fungal content, but not significantly less than inbred lines CKL05015 (0.007 ng uL^{-1}), CKL05003 (0.008 ng uL^{-1}), CB 222 (0.009 ng uL^{-1}), US 2540W (0.010 ng uL^{-1}) and CKL05022 (0.010 ng uL^{-1}) (Table 3). Lines R2565y (0.271 ng uL^{-1}), R119W (0.110 ng uL^{-1}) and I137tnW (0.048 ng uL^{-1}) had the highest fungal content, and differed significantly from each other. At Vaalharts, R2565y (0.019 ng uL^{-1}), CML 495 (0.026 ng uL^{-1}), CKL05015 0.028 ng uL^{-1}), RO 549W (0.029 ng uL^{-1}) and CML 182 (0.031 ng uL^{-1}) were the least and CML 444 (0.343 ng uL^{-1}) the most contaminated with *F. verticillioides* (Table 3).

In Kenya, inbred lines CML 495 (0.000 ng uL^{-1}) and CML 444 (0.000 ng uL^{-1}) were resistant to *F. verticillioides* colonisation, accumulating the least fungal target DNA in Katumani and Kiboko, respectively (Table 3). Their fungal content levels, however, did not differ significantly from MIRTC5 (0.000 ng uL^{-1}), CML 264 (0.000 ng uL^{-1}), CKL05019 (0.000 ng uL^{-1}) and CKL05022 (0.018 ng uL^{-1}) at Katumani, or from CKL05015 (0.117 ng uL^{-1}) and CML 247 (0.132 ng uL^{-1}) at Kiboko. South African lines US 2540W (0.453 ng uL^{-1}), R119W (0.287 ng uL^{-1}) and CML 390 (0.280 ng uL^{-1}) were the most susceptible to *F. verticillioides* colonisation at Katumani, whereas I137tnW (4.587 ng uL^{-1}) was the most susceptible at Kiboko. Lines R119W (0.979 ng uL^{-1}), CB 248 (0.644 ng uL^{-1}) and R2565y (0.628 ng uL^{-1}) accumulated significantly less fungal target DNA than the most susceptible inbred line I137tnW (4.587 ng uL^{-1}), but significantly more than the most resistant lines CML 444 (0.116 ng uL^{-1}), CKL05015 (0.117 ng uL^{-1}), CML 495 (0.124 ng uL^{-1}) and CML 247 (0.132 ng uL^{-1}) at Kiboko (Table 3). Across localities, R119W (0.139 ng uL^{-1}) was significantly more susceptible to fungal colonisation than R2565y (0.076 ng uL^{-1}) and CML 247 (0.066 ng uL), which did not differ significantly from one another. Line CML 495 (0.012 ng uL^{-1}) was most resistant to fungal contamination, but not significantly more than inbred lines RO 549W (0.017 ng uL^{-1}), MIRTC5 (0.017 ng uL^{-1}), LaPosta (0.019 ng uL^{-1}), CKL05019 (0.019 ng uL^{-1}) and CML 182 (0.021 ng uL^{-1}), CKL05003 (0.24 ng uL^{-1}), CKL05022 (0.24 ng uL^{-1}), CML 264 (0.24 ng uL^{-1}), VO 617y-2 (0.26 ng uL^{-1}) and CB-222 (0.31 ng uL^{-1}), (Table 3).

Grain colonisation by *F. verticillioides* was significantly ($P \leq 0.05$) influenced by the inbred line (genotype), locality (environment) and the interaction between the two (Table 5). The G x E interaction was responsible for 52.7% of the variation, while the environment and genotype accounted for 31.8 and 15.4% of the variation, respectively. Further analysis of the GEI revealed that IPCA 1 and IPCA2 were both significant and explained 46.6 and 4.2% of the variation observed, respectively. In total the IPCAs accounted for 50.8% of the total G x E variation influencing fungal colonisation. The stability of inbred line response to *F. verticillioides* colonisation, as determined by the ASV, ranged between 0.13 and 11.88 with a mean of 1.61 (Table 6). Inbred line RO 549W (0.13), P502c2 (0.18), CKL05019 (0.25),

CKL05022 (0.26) and CML 264 (0.45) were the most stable in their response to fungal colonisation across localities, while I137tnW (11.88) was the least stable (Table 6).

The first two principal components of the GGE biplot analysis accounted for 95.2% (PC1: 87.2% and PC2: 8.0%) of the total GGE variation for fungal colonisation (Fig. 2). Genotype ranking and genotype comparison biplots were congruent in determining CML 495 (#12), CKL05019 (#5) and CML 444 (#11) as ideal genotypes with stable resistance to fungal colonisation across localities, while I137tnW (#13) was the worst performing genotype (Fig. 2A and B). Inbred line CML 444 (#11) was the best performing genotype across all environments, resulting in all the localities constituting a single mega-environment (Fig. 2C). Other inbred lines that were also resistant across localities included CML 495 (#12), CKL05019 (#5), CML 264 (#9) and CML 247 (#8) and CKL05022 (#6). Vertex genotypes CML 444 (#11), CKL05015 (#4), US 2540W (#20) and I137tnW (#13) delimited the polygon and differed most in their response to fungal colonisation (Fig. 2C). Environment-comparison biplot revealed Kiboko as the ideal test environment, followed by Katumani, Makhatini and Potchefstroom (Fig. 2D).

Fumonisin accumulation

The average fumonisin content in maize grain from inbred lines evaluated at Makhatini (5.0 mg kg^{-1}) and Vaalharts (5.2 mg kg^{-1}) did not differ significantly, but was significantly more than the fumonisin contamination observed at Potchefstroom (1.4 mg kg^{-1}). Fumonisin contamination of inbred lines evaluated in South Africa was significantly less than those evaluated in Kenya. Significantly more fumonisin also accumulated in maize grain collected at Kiboko (37.0 mg kg^{-1}) than at Katumani (8.9 mg kg^{-1}) (Table 4).

RO 549W (3.0 mg kg^{-1}) accumulated the least fumonisins at Makhatini, but its fumonisin content differed significantly only from R119W (10.4 mg kg^{-1}), VO 617y-2 (8.1 mg kg^{-1}) and CB 248 (6.3 mg kg^{-1}), which were most contaminated with fumonisins (Table 4). At Potchefstroom, inbred lines CML 495 (0.4 mg kg^{-1}), CML 390 (0.4 mg kg^{-1}), CKL05015 (0.4 mg kg^{-1}), CKL05022 (0.5 mg kg^{-1}), US 2540W (0.5 mg kg^{-1}), CKL05003 (0.5 mg kg^{-1}), MIRTC5 (0.6 mg kg^{-1}), RO 549W (0.6 mg kg^{-1}) and CB 222 (0.6 mg kg^{-1}) were significantly less contaminated with fumonisins compared to R119W (7.8 mg kg^{-1}), R2565y (5.5 mg kg^{-1}), I137tnW (3.0 mg kg^{-1}) and CB 248 (1.8 mg kg^{-1}). All inbred lines evaluated at Vaalharts were significantly less contaminated with fumonisins when compared to CML 444 (28.7 mg kg^{-1}), P502c2 (12.9 mg kg^{-1}) and CKL05019 (6.7 mg kg^{-1}) (Table 4). In Kenya, lines R119W (23.5 mg kg^{-1}), CB 222 (23.4 mg kg^{-1}) and R2565y (19.2 mg kg^{-1}) were most contaminated with fumonisins at Katumani, and MIRTC5 (3.5 mg kg^{-1}), CML 444 (3.5 mg kg^{-1}), CKL05019 (3.7 mg kg^{-1}), CML 264 (3.7 mg kg^{-1}) and P502c2 (3.7 mg kg^{-1}) the least contaminated (Table 4). At Kiboko, inbred lines I137tnW (100.2 mg kg^{-1}), P502c2 (92.2 mg kg^{-1}), R2565y (66.8 mg kg^{-}

^{1}) and R119W (66.6 mg kg^{-1}) were significantly more contaminated with fumonisins than CML 182 (6.1 mg kg^{-1}), CKL05015 (9.2 mg kg^{-1}) and CML 495 (13.0 mg kg^{-1} (Table 4). Inbred lines RO 549W (1.9 mg kg^{-1}), CML 495 (2.0 mg kg^{-1}) and MIRTC5 (2.1 mg kg^{-1}) were the least and R119W the most contaminated with fumonisins across locations (Table 4).

The environment, genotype and the interaction between them ($P \leq 0.05$) had a highly significant effect on the total fumonisins that accumulated in the grain of maize inbred lines (Table 5). The largest source of variation in fumonisin contamination was the environment, and it explained 71.9% of the total variation. The genotype and GEI further accounted for 8.4 and 19.7% of the variation, respectively. The IPCA1 and IPCA 2 were both significant and explained 9.8 and 7.7% of the total variation, respectively (Table 5). The stability of inbred line response to fumonisin accumulation across environments was determined by ASV, which ranged from 0.23 to 1.35 (Table 6). Inbred line CKL05003 (0.23), LaPosta (0.24), MIRTC5 (0.24), CML 495 (0.26), US 2540W (0.27), CKL05022 (0.32) and CML 264 (0.32) were most stable in their response to fumonisin accumulation across localities, while CML 444 (1.35) and P502c2 (1.08) were the least stable (Table 6).

The principal components of the GEI affecting fumonisin accumulation accounted for 79.8% (PC1: 45.1% and PC2: 34.7%) of the total GGE variation (Fig. 3A). Inbred line CML 247 (#8) was the most resistant to fumonisin accumulation, along with CKL05003 (#3) and CML 495 (#12). The ideal genotype based on mean and stability in the genotype-comparison biplot revealed CML 247 (#8) as the ideal genotype, while CKL05003 (#3) and CML 495 (#12) were desirable due to their proximity to CML 247 (Fig. 3B). The inbred lines CML 444 (#11), CKL05015 (#4), R2565y (#18), R119W (#17), I137tnW (#13) and P502c2 (#16) formed the polygon and differed most in their response to fumonisin contamination (Fig. 3C). Line CML 444 (#11) was the best performing genotype at Katumani, Makhatini and Potchefstroom, which resulted in a mega-environment for this inbred line. Line CKL05015 (#4) was the best performing genotype at Kiboko, while R2565y (#18) was the best performing genotype at Vaalharts. The localities evaluated were divided into different sectors, indicating significant crossover interaction, with Kiboko being the ideal test environment (Fig. 3D).

Correlations between FER symptoms, *Fusarium verticillioides* target DNA and total fumonisins

Significant correlations were obtained between FER severity, fungal colonisation and total fumonisin content at all the test localities. Disease severity had a significant but moderate correlation with *F. verticillioides* target DNA ($R = 0.54$; $P < 0.0001$), and a significantly weak relationship with total fumonisins ($R = 0.38$; $P = 0.0019$) at Makhatini. Fungal target DNA and fumonisins showed a significantly strong correlation ($R = 0.66$; $P < 0.0001$) (Fig. 4A-C). At

Potchefstroom, a significant but moderate correlation was found between FER severity and fungal target DNA (R = 0.58; P < 0.0001), while a significantly strong correlation was obtained between disease severity and total fumonisins (R = 0.88; P < 0.0001), as well as between fungal target DNA content and total fumonisin content (R = 0.76; P < 0.0001) (Fig. 5A-C). Significantly strong relationships were found at Vaalharts between FER severity and *F. verticillioides* target DNA (R = 0.82; P < 0.0001), disease severity and total fumonisin content (R = 0.77; P < 0.0001), as well as between fungal target DNA and total fumonisins (R = 0.81; P < 0.0001) (Fig. 6A-C).

FER severity at Katumani had a significant though moderate correlation with fungal target DNA content (R = 0.40; P=0.0011) and total fumonisin content (R = 0.39; P = 0.0015). The *F. verticillioides* content of inbred lines also had a significant but moderate correlation with total fumonisin content (R = 0.55; P < 0.0001) (Fig. 7A-C). At Kiboko, significant but moderate relationships were obtained between FER severity and fungal target DNA (R = 0.56; P < 0.0001), disease severity and total fumonisin content (R = 0.55; P < 0.0001) and fungal target DNA content and total fumonisins (R = 0.58; P < 0.0001) (Fig. 8A-C). Across all localities, significant but moderately strong correlations were found between FER severity and *F. verticillioides* target DNA (R = 0.63; P < 0.0001), disease severity and total fumonisin content (R = 0.63; P < 0.0001), as well as between fungal target DNA content and total fumonisins (R = 0.67; P < 0.0001) (Fig. 9A-C).

DISCUSSION

This study investigated Kenyan maize inbred lines, previously characterised for resistance to *A. flavus* and aflatoxin contamination, for *F. verticillioides* resistance for the first time, both under South African and Kenyan conditions. The Kenyan maize inbred line CML 495 was found to be more resistant to FER, *F. verticillioides* colonisation and fumonisin accumulation across environments than any of the South African inbred lines previously characterised in South Africa. The inbred line's performance was stable across locations, even when evaluated under temperate conditions when compared to its adapted tropical and subtropical conditions. Therefore, CML 495 is also considered to have broad adaptability. It has also been characterised as a late maturing line (CIMMYT, 2005), which dries down slower than early maturing lines, thereby potentially extending the conditions conducive for fumonisin deposition. Nonetheless, CML 495 demonstrated superior resistance to *F. verticillioides* infection and fumonisins. The maturity class of hybrids had the greatest effect on fumonisin contamination in a study in northern Italy (Battilani *et al*., 2008) while other studies noted that the maturity class did not influence the final fumonisin concentration in maize kernels (Ramirez *et al*., 1996; Blandino *et al*., 2009; Battilani *et al*., 2011). Hybrids with the slowest

kernel drying rate, however, were most favourable for fumonisin accumulation irrespective of the maturity classification (Battilani *et al*., 2011). The use of CML 495 in breeding programmes would not only provide a source of resistance to *A. flavus* and aflatoxins, but provide comprehensive resistance to *F. verticillioides* and fumonisin contamination too.

Two additional Kenyan inbred lines, CML 264 and CKL05015, also developed less than 5% FER, with grain containing less than 0.05 ng μL^{-1} fungal target DNA and less than 3.0 mg kg^{-1} fumonisins. Inbred line CML 264 was more stable in its response to disease expression, fungal colonisation and fumonisin accumulation. Inbred line CKL05015 was less stable in its response to FER severity across localities and showed specific adaptation at localities like Kiboko and Vaalharts where it showed resistance to fungal and fumonisin contamination. Inbred lines resistant to AER and aflatoxin accumulation, thus, appeared to be good sources of resistance to *F. verticillioides* and fumonisin accumulation. The identification of new sources of resistance to FER and fumonisin accumulation is of great importance, as all cultivars grown in South Africa are highly susceptible to FER and fumonisin contamination, with fumonisin levels often exceeding the tolerable limits enforced in Europe and the USA (Rheeder *et al*., 1990; Janse van Rensburg *et al*., 2015).

The response of inbred lines to FER, *F. verticillioides* colonisation and total fumonisin content was not always congruent. Inbred lines CKL05019, LaPosta, CB 222 and CML 444; which were all highly susceptible to FER, were intermediately resistant to fungal colonisation and fumonisins contamination. Inbred line CML 247, which was intermediately resistant to FER, was susceptible to fungal colonisation but accumulated low levels of fumonisins. These results suggest that the genetic potential for resistance to FER (disease expression), fungal colonisation and fumonisin accumulation may exist independently from one another and is predominantly determined by the genetic composition of the maize line and the environment.

GEI significantly affected the performance of all inbred lines evaluated in this study. The GEI contributed more to the variation in FER severity and fungal colonisation than the genotype or environment, while the environment contributed the most to the variation observed in fumonisin content. These results indicate that inbred line response to *F. verticillioides* infection is dependent on the plant genotype to resist or succumb to infection under prevailing environmental conditions that support fungal development, inoculum pressure and insect infestation (Munkvold, 2003).

The significant influence of the environment on fumonisin accumulation indicates the need to determine the suitability of an environment to promote the biosynthesis of fumonisins by *F. verticillioides*. Differential response of inbred lines to FER and fumonisin accumulation due to GEI has previously been documented (Clements and White, 2004; Afolabi *et al*., 2007; Small *et al*., 2012), while fumonisin contamination was shown to be predominantly affected by the prevailing environmental conditions (Cao *et al*., 2014). This was also

observed by de la Campa *et al*. (2005) when modelling the effects of environment, insect damage and *Bt* genotypes on fumonisin accumulation. The strong influence of environment on fumonisin accumulation accentuates the value of evaluating resistance to fumonisin accumulation in target environments. Fumonisin accumulation of maize inbred lines was particularly severe in Kiboko, Kenya. The contamination levels of maize grain obtained in this location far exceeded the 4 mg kg^{-1} fumonisin allowed for human and animal consumption as set by United States authorities (FDA, 2000). The region is characterised by a bimodal rainfall with long rains occurring in March to May and short rains occurring from October to December/January. In 2013 the mean rainfall of 107.5 mm and 54.5 mm recorded at Kiboko during the long and short rainfall period, respectively, differed significantly from Katumani. The rainfall coincided with the grain filling period, while mean maximum temperature at Kiboko (31.3°C) also differed significantly from Katumani (25.5°C), creating a favourable environment for fumonisin production at Kiboko (Janse van Rensburg, 2011). Temperature was shown to play a significant role in fumonisin production during the dough-stage of grain fill (de la Campa *et al*., 2005; Janse van Rensburg, 2011) while drought-stress significantly affected FER and fumonisins (Parson and Munkvold, 2010). Kiboko provides the ideal environment for evaluating maize genotypes for resistance to *F. verticillioides* and more specifically fumonisin accumulation.

Significant, though inconsistent relationships between FER, *F. verticillioides* target DNA and total fumonisin content were obtained in this study. Significantly moderate correlations were obtained between FER and fungal target DNA at all localities except Vaalharts where a strong, positive correlation was obtained. Poor to moderate correlations were obtained between FER and fumonisin content at Makhatini, Katumani and Kiboko while strong, positive correlations were obtained at Potchefstroom and Vaalharts. The relationship between fungal and fumonisin contamination was moderate to strong at all the localities. These results demonstrate the inconsistency of the relationship between FER/fungal target DNA and FER/fumonisins in selecting for reduced fumonisins, as they were significantly influenced by the production season (prevailing weather conditions and trial management practises). The relationship between fungal target DNA and fumonisins provided a more accurate indication of potential resistance to fumonisin accumulation (Janse van Rensburg *et al*., 2015). The inbred lines CKL05019, CB 222, LaPosta and CML 444 were highly susceptible to FER, with little fumonisin contamination, which reduced the relationship between these variables. These results emphasize the need for fumonisin analysis when evaluating resistance to FER and fumonisins (Afolabi *et al*., 2007; Small *et al*., 2012). The reduced relationship between FER severity, *F. verticillioides* and fumonisins could also be due to

inaccurate visual assessment of disease symptoms and the ability of other *Fusarium* species to cause maize ear rot diseases. The cost and expertise required to quantify fungal target DNA or fumonisins may, however, be a deterrent in their implementation in resistance breeding programmes.

The use of resistant cultivars is considered the most economically viable, environmentally sound and convenient for producers to manage fumonisin contamination (Clements and White, 2004). In this study, inbred lines resistant to AER and aflatoxin accumulation were more resistance to FER and fumonisin accumulation across localities when compared to South African FER/fumonisin-resistant inbred lines. These lines provide additional sources of resistance that could be used to improve resistance to FER and fumonisin in elite local lines or be used for the development of hybrids with improved resistance. The inbred lines are also a valuable resource that could be utilised in genomic and proteomic approaches to understand resistance mechanisms toward AER, FER and their associated mycotoxins.

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Table 1. Maize inbred lines evaluated for resistance to Fusarium ear rot, *Fusarium verticillioides* colonisation and fumonisin accumulation in South Africa and Kenya during the 2012/13 maize growing seasons.

Number[1]	Inbred name	Origin[2]	FER/fumonisin status[3]	AER/aflatoxin status
1	**CB-222**	ARC-GCI- South Africa	Resistant	Unknown
2	**CB-248**	ARC-GCI- South Africa	Resistant	Unknown
3	**CKL05003**	CIMMYT-Kenya	Unknown	Resistant
4	**CKL05015**	CIMMYT-Kenya	Unknown	Resistant
5	**CKL05019**	CIMMYT-Kenya	Unknown	Resistant
6	**CKL05022**	CIMMYT-Kenya	Unknown	Resistant
7	**CML 182**	CIMMYT-Zimbabwe	Resistant	Unknown
8	**CML 247**	CIMMYT-Kenya	Unknown	Resistant
9	**CML 264**	CIMMYT-Kenya	Unknown	Resistant
10	**CML 390**	ARC-GCI- South Africa	Resistant	Unknown
11	**CML 444**	ARC-GCI- South Africa	Resistant	Unknown
12	**CML 495**	CIMMYT-Kenya	Unknown	Resistant
13	**I137tnW**	ARC-GCI- South Africa	Susceptible	Unknown
14	**La Posta**	CIMMYT-Kenya	Unknown	Resistant
15	**MIRTC5**	CIMMYT-Kenya	Unknown	Resistant
16	**P502c2**	CIMMYT-Kenya	Unknown	Resistant
17	**R119W**	ARC-GCI- South Africa	Resistant	Unknown
18	**R2565y**	CIMMYT-Zimbabwe	Susceptible	Unknown
19	**RO 549W**	CIMMYT-Zimbabwe	Resistant	Unknown
20	**US 2540W**	ARC-GCI- South Africa	Resistant	Unknown
21	**VO 617y-2**	CIMMYT-Zimbabwe	Resistant	Unknown

[1]Designated line number of the inbred lines used in this study
[2]Agricultural Research Council – Grain Crops Institute (ARC-GCI), CIMMYT – International Maize and Wheat Improvement Center
[3]According to Small *et al*. (2012) and Mouton (2014)

Table 2. Mean Fusarium ear rot of maize inbred lines evaluated in South Africa and Kenya during the 2012/13 maize growing seasons.

	***Fusarium ear rot severity (%)*[1,2]**					
	South Africa			**Kenya**		
Inbred line	*Makhatini*	*Potchefstroom*	*Vaalharts*	*Katumani*	*Kiboko*	*Across localities*
CB-222	8.5 b	4.0 d-f	2.9 ed	23.8 a	20.0 c-f	**10.2 bc**
CB-248	1.8 b-d	9.1 cd	2.5 ed	9.5 bc	44.6 bc	**6.3 b-g**
CKL05003	3.9 b-d	3.4 d-f	8.3 c-e	4.7 bc	39.7 b-d	**6.3 b-g**
CKL05015	1.1 cd	0.4 f	1.8 e	10.4 bc	0.6 f	**3.3 fg**
CKL05019	5.0 b-d	3.3 d-f	19.9 b	1.3 bc	6.7 ef	**7.8 b-f**
CKL05022	2.5 b-d	3.4 d-f	14.3 bc	0.9 c	11.9 d-f	**5.7 b-g**
CML 182	0.5 d	5.4 c-f	3.9 c-e	8.5 bc	33.0 c-e	**5.1 d-g**
CML 247	5.5 b-d	8.1 c-e	9.3 c-e	1.9 bc	3.3 ef	**6.0 b-g**
CML 264	0.3 d	1.4 ef	6.2 c-e	0.8 c	11.1 d-f	**2.5 g**
CML 390	7.6 bc	1.1 ef	4.0 c-e	8.6 bc	5.1 ef	**5.7 b-g**
CML 444	0.2 d	3.1 d-f	32.6 a	0.0 c	3.3 ef	**9.4 b-d**
CML 495	0.6 cd	4.5 d-f	2.9 ed	0.6 c	3.6 ef	**2.0 g**
I137TNW	28.2 a	23.3 b	10.4 b-e	5.7 bc	85.6 a	**19.0 a**
La Posta	2.9 b-d	4.7 d-f	7.4 c-e	25.3 a	28.5 c-f	**10.5 b**
MIRTC5	2.0 b-d	12.6 c	5.4 c-e	1.4 bc	11.3 d-f	**4.9 d-g**
P502c2	2.7 b-d	1.6 d-f	12.4 b-d	1.3 bc	38 b-d	**5.9 b-g**
R119W	26.8 a	46.2 a	9.0 c-e	2.1 bc	65.8 ab	**20.8 a**
R2565y	4.5 b-d	21.4 b	0.5 e	10.5 bc	20.8 c-f	**8.3 b-e**
RO 549W	2.1 b-d	5.3 c-f	0.3 e	3.9 bc	32.0 c-e	**3.5 e-g**
US 2540W	1.6 b-d	3.2 d-f	3.4 ed	14.0 ab	4.3 ef	**5.4 c-g**
VO 617y-2	0.0 d	1.6 d-f	6.0 c-e	0.2 c	24.4 c-f	**2.7 g**
Mean	***5.2 b***	***8.0 b***	***7.8 b***	***6.4 b***	***23.5 a***	***7.2***

[1]The percentage of maize ears covered with visual symptoms of Fusarium ear rot, represented by the mean of three trial plots.
[2]Means followed by the same alphabetical letter in each column are not significantly different according to the Student's t-test of least significant differences ($P=\leq 0.05$).

Table 3. *Fusarium verticillioides* colonisation of grain collected from maize inbred lines evaluated in South Africa and Kenya during the 2012/13 maize growing seasons.

***F. verticillioides* DNA (ng uL^{-1})[1,2]**						
	South Africa			***Kenya***		
Inbred line	*Makhatini*	*Potchefstroom*	*Vaalharts*	*Katumani*	*Kiboko*	*Across localities*
CB-222	0.023 c-h	0.009 ef	0.062 cd	0.290 ab	0.356 c-f	**0.031 d-h**
CB-248	0.034 c-f	0.015 d-f	0.066 cd	0.198 bc	0.644 bc	**0.038d-f**
CKL05003	0.015 e-h	0.008 ef	0.102 b-d	0.131 b-d	0.151 ef	**0.024 e-h**
CKL05015	0.040 c	0.007 ef	0.028 d	0.178 b-d	0.117 f	**0.037 d-g**
CKL05019	0.009 gh	0.015 d-f	0.114 b-d	0.000 d	0.418 c-f	**0.019 f-h**
CKL05022	0.013 f-h	0.01 d-f	0.148 bc	0.018 d	0.349 c-f	**0.024 e-h**
CML 182	0.019 d-h	0.02 d-f	0.031 d	0.062 cd	0.143 ef	**0.021 f-h**
CML 247	0.063 b	0.03 cd	0.175 b	0.049 cd	0.132 f	**0.066 bc**
CML 264	0.022 c-h	0.017 d-f	0.06 cd	0.000 d	0.448 c-f	**0.024 e-h**
CML 390	0.029 c-g	0.005 f	0.084 b-d	0.280 ab	0.154 ef	**0.036 d-g**
CML 444	0.007 h	0.012 d-f	0.343 a	0.000 c	0.116 f	**0.035 d-g**
CML 495	0.013 gh	0.003 f	0.026 d	0.000 c	0.123 f	**0.012 h**
I137TNW	0.027 c-h	0.048 c	0.074 b-d	0.056 cd	4.587 a	**0.039 d-f**
La Posta	0.012 gh	0.012 d-f	0.075 b-d	0.053 cd	0.262 c-f	**0.019 f-h**
MIRTC5	0.014 f-h	0.011 d-f	0.066 cd	0.000 c	0.209 d-f	**0.017 gh**
P502c2	0.037 cd	0.018 d-f	0.146 bc	0.048 cd	0.433 c-f	**0.043 de**
R119W	0.139 a	0.11 b	0.146 bc	0.287 ab	0.979 b	**0.139 a**
R2565y	0.029 c-g	0.271 a	0.019 d	0.166 b-d	0.628 b-d	**0.076 b**
RO 549W	0.011 gh	0.026 de	0.029 d	0.061 cd	0.328 c-f	**0.017 gh**
US 2540W	0.035 c-e	0.01 d-f	0.119 b-d	0.453 a	0.333 c-f	**0.049 cd**
VO 617y-2	0.017 d-h	0.026 de	0.092 b-d	0.075 cd	0.194 ef	**0.026 e-h**
Mean	***0.029 c***	***0.033 c***	***0.095 bc***	***0.115 b***	***0.529 a***	***0.038***

[1]The mean of *F. verticillioides* DNA (ng uL^{-1}) quantified in three field plots.

[2]Means followed by the same alphabetical letter in each column are not significantly different according to the Student's t-test of least significant differences (P=≥ 0.05).

Table 4. Total fumonisin content in grain of maize inbred lines evaluated in South Africa and Kenya during the 2012/13 maize growing seasons.

	Total fumonisins (mg kg^{-1})[1,2]					
	South Africa			Kenya		
Inbred line	*Makhatini*	*Potchefstroom*	*Vaalharts*	*Katumani*	*Kiboko*	*Across localities*
CB-222	5.0 b-d	0.6 e	3.1 cd	23.4 a	25.0 e-h	**3.2 c-g**
CB-248	6.3 bc	1.8 d	1.4 d	9.8 b-d	47.6 c-f	**3.8 cd**
CKL05003	3.9 cd	0.5 e	2.7 cd	6.5 cd	15.4 f-h	**2.1 e-g**
CKL05015	5.2 b-d	0.4 e	1.5 d	11 b-d	9.1 gh	**2.7 d-g**
CKL05019	3.7 cd	0.9 de	6.7 c	3.7 d	25.5 e-h	**2.4 d-g**
CKL05022	3.7 cd	0.5 e	5.0 cd	5.7 cd	34.8 c-h	**2.2 e-g**
CML 182	4.5 cd	0.9 de	2.7 cd	11.7 b-d	6.1 h	**2.8 d-g**
CML 247	6.0 b-d	1.0 de	5.6 cd	7.2 cd	14.9 f-h	**3.4 c-f**
CML 264	3.9 cd	0.8 de	3.1 cd	3.7 d	34.5 c-h	**2.2 e-g**
CML 390	4.7 b-d	0.4 e	2.7 cd	14.1 a-c	23.3 e-h	**2.6 d-g**
CML 444	4.0 cd	0.8 de	28.7 a	3.5 d	14.2 f-h	**3.5 c-e**
CML 495	3.9 cd	0.4 e	1.6 d	4.6 cd	13.0 f-h	**2.0 fg**
I137TNW	5.6 b-d	3.0 c	4.0 cd	4.8 cd	100.2 a	**4.3 bc**
La Posta	5.6 b-d	0.9 de	5.7 cd	5.8 cd	43.2 c-g	**3.2 c-g**
MIRTC5	3.8 cd	0.6 e	3.4 cd	3.5 d	27.4 d-h	**2.1 e-g**
P502c2	4.4 cd	0.7 de	12.9 b	3.7 d	92.2 ab	**3.0 c-g**
R119W	10.4 a	7.8 a	4.5 cd	23.5 a	66.5 a-c	**9.3 a**
R2565y	4.7 cd	5.5 b	1.4 d	19.2 ab	66.8 a-c	**5.6 b**
RO 549W	3.0 d	0.6 e	4.5 cd	4.8 cd	26.2 d-h	**1.9 g**
US 2540W	4.4 cd	0.5 e	2.8 cd	11.7 b-d	30.4 d-h	**2.5 d-g**
VO 617y-2	8.1 ab	1.0 de	4.4 cd	6.0 cd	61.3 b-d	**4.2 bc**
Mean	***5.0 c***	***1.4 d***	***5.2 c***	***8.9 b***	***37.0 a***	***3.3***

[1]The mean of fumonisin content represented as the sum of FB_1, FB_2 and FB_3 (mg kg^{-1}) for three trial plots

[2]Means followed by the same alphabetical letter in each column are not significantly different according to the Student's t-test of least significant differences ($P \leq 0.05$).

Table 5. Additive main effects and multiplicative interaction analysis of variance of Fusarium ear rot (FER), *Fusarium verticillioides* colonisation and total fumonisin content of maize inbred lines evaluated in South Africa and Kenya.

		FER severity[1]				*F. verticillioides* colonisation[2]				Fumonisin accumulation[3]			
Source of variation	***d.f.***	***s.s.***	***m.s.***	***F pr***	***Variation (%)***	***s.s.***	***m.s.***	***F pr***	***Variation (%)***	***s.s.***	***m.s.***	***F pr***	***Variation (%)***
Total	314	500	1.592			13.801	0.044			350.6	1.117		
Treatments	104	337.4	3.244	<0.001		12.343	0.1187	<0.001		312	3	<0.001	
Genotypes	20	84.4	4.218	<0.001	25.0	1.906	0.0953	<0.001	15.4	26.1	1.304	<0.001	8.4
Environments	4	57.7	14.425	<0.001	17.1	3.931	0.9828	<0.001	31.8	224.4	56.104	<0.001	71.9
Block	10	8.2	0.825	0.3884		0.039	0.0039	0.8535		1.1	0.107	0.8357	
Interactions	80	195.3	2.442	<0.001	57.9	6.506	0.0813	<0.001	52.7	61.5	0.769	<0.001	19.7
IPCA 1	23	85.8	3.73	<0.001	25.4	5.752	0.2501	<0.001	46.6	30.5	1.325	<0.001	9.8
IPCA 2	21	57.7	2.749	<0.001	17.1	0.514	0.0245	<0.001	4.2	23.9	1.136	<0.001	7.7
Residuals	36	51.8	1.439	0.0039		0.24	0.0067	0.5696		7.2	0.199	0.3873	
Error	200	154.4	0.772			1.418	0.0071			37.5	0.188		

[1]The percentage of maize ears covered with visual symptoms of Fusarium ear rot, [2]The absolute concentrations of *F. verticillioides* target DNA and [3]Total fumonisin content as the sum of FB_1, FB_2 and FB_3.

[2]Natural log transformation used in analyses

[3]IPCA – Interaction Principle Component Axis scores

Table 6. Additive main effects and multiplicative interaction analysis stability values (ASVs) and interaction principle component axes (IPCA) values for Fusarium ear rot (FER), *Fusarium verticillioides* colonisation and total fumonisin content of maize inbred lines evaluated in South Africa and Kenya.

	FER severity[1]			***F. verticillioides* colonisation**[2]			**Fumonisin accumulation**[3]		
Genotype	*IPCA1*	*IPCA2*	*ASV*	*IPCA1*	*IPCA2*	*ASV*	*IPCA1*	*IPCA2*	*ASV*
CB-222	0.6	-0.54	1.04	0.05	-0.22	0.58	-0.38	-0.18	0.51
CB-248	0.71	0.21	1.08	-0.11	-0.12	1.19	-0.37	0.23	0.53
CKL05003	0.3	0.27	0.52	0.15	-0.03	1.64	-0.1	-0.19	0.23
CKL05015	0.01	-1.12	1.12	0.17	-0.13	1.86	-0.48	-0.47	0.77
CKL05019	-0.85	-0.18	1.27	-0.02	0.14	0.25	0.35	-0.12	0.46
CKL05022	-0.69	0.03	1.02	0.02	0.14	0.26	0.25	0.05	0.32
CML 182	0.58	0.03	0.87	0.13	0	1.41	-0.43	-0.64	0.84
CML 247	-0.55	-0.05	0.83	0.17	0.13	1.88	0	-0.48	0.48
CML 264	-0.19	0.21	0.35	-0.04	0.1	0.45	0.18	0.22	0.32
CML 390	0.12	-0.66	0.68	0.17	-0.19	1.87	-0.27	-0.15	0.37
CML 444	-1.14	0.06	1.69	0.19	0.29	2.1	0.89	-0.73	1.35
CML 495	-0.31	-0.09	0.46	0.12	0.07	1.37	-0.15	-0.18	0.26
I137TNW	0.18	0.63	0.69	-1.06	0.04	11.88	0.21	0.7	0.75
LAPOSTA	0.31	-0.27	0.54	0.06	0.04	0.63	0.13	0.17	0.24
MIRTC5	-0.22	0.31	0.45	0.08	0.1	0.86	0.17	0.11	0.24
P502c2	-0.26	0.29	0.48	-0.01	0.1	0.18	0.8	0.36	1.08
R119W	0.23	0.86	0.92	-0.19	-0.12	2.16	-0.41	0.29	0.6
R2565Y	0.6	-0.24	0.92	-0.06	-0.08	0.72	-0.59	0.56	0.94
RO 549W	0.66	0.38	1.05	0.01	0.02	0.13	0.29	0.05	0.38
US 2540W	0.05	-0.6	0.61	0.09	-0.32	1.07	-0.21	0.03	0.27
VO 617y-2	-0.15	0.48	0.53	0.11	0.04	1.22	0.11	0.35	0.38
Mean			***0.82***			***1.61***			***0.54***

[1]The percentage of maize ears overed with visual symptoms of Fusarium ear rot
[2]The absolute concentrations of *F. verticillioides* target DNA
[3]Total fumonisin content as the sum of FB_1, FB_2 and FB_3 for three field plots

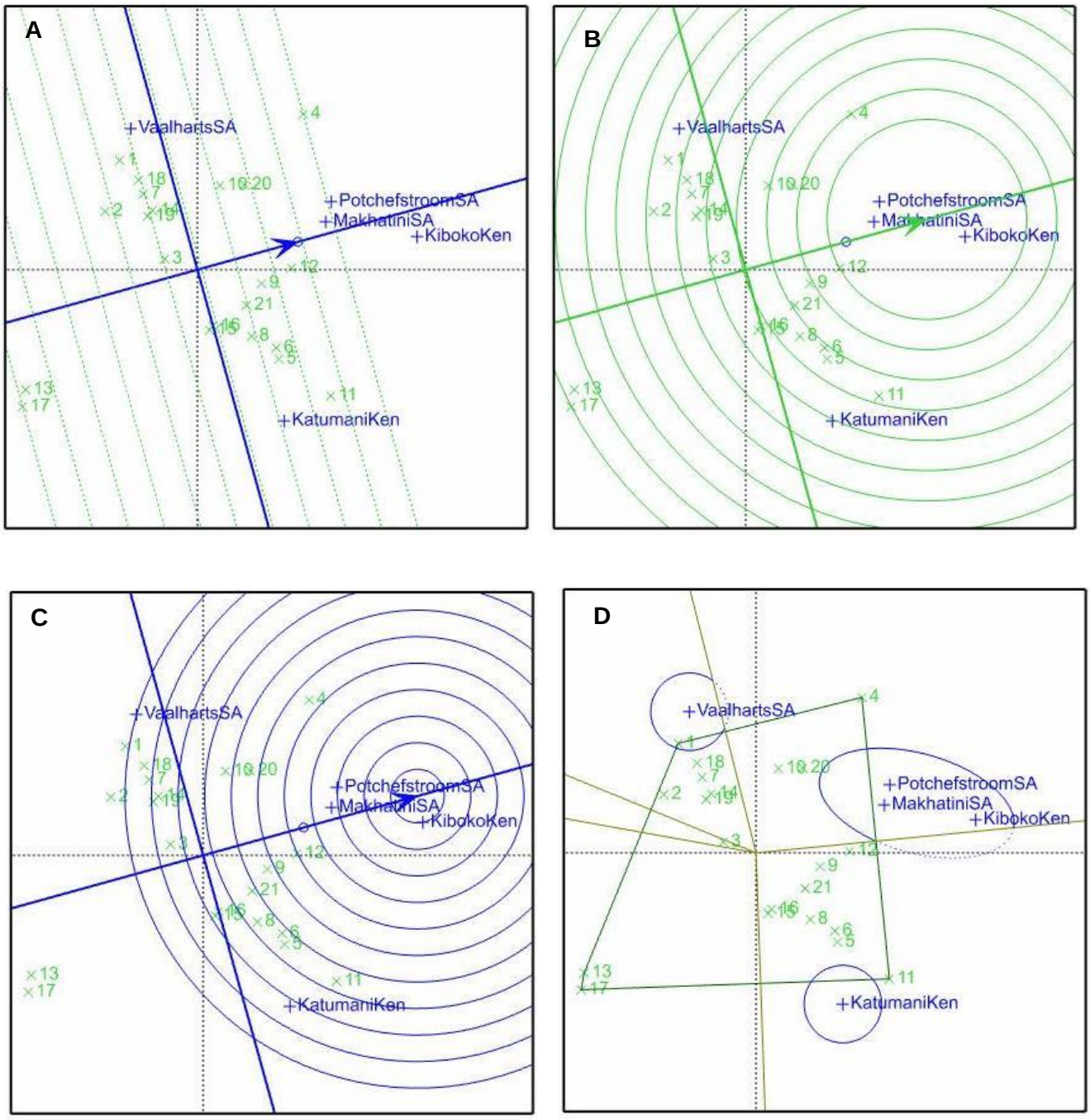

Figure 1. Genotype main effect and genotype by environment interaction biplot for Fusarium ear rot severity in 21 maize inbred lines tested, based on environment-focused scaling for **A.** the genotype ranking, **B.** genotype comparison, **C.** environment comparison and **D.** the polygon view exhibiting mega-environments. Green and blue numbers represent genotypes and environments, respectively, with localities represented as: Potchefstroom (SA), Makhatini (SA), Kiboko (KEN) and Katumani (KEN).

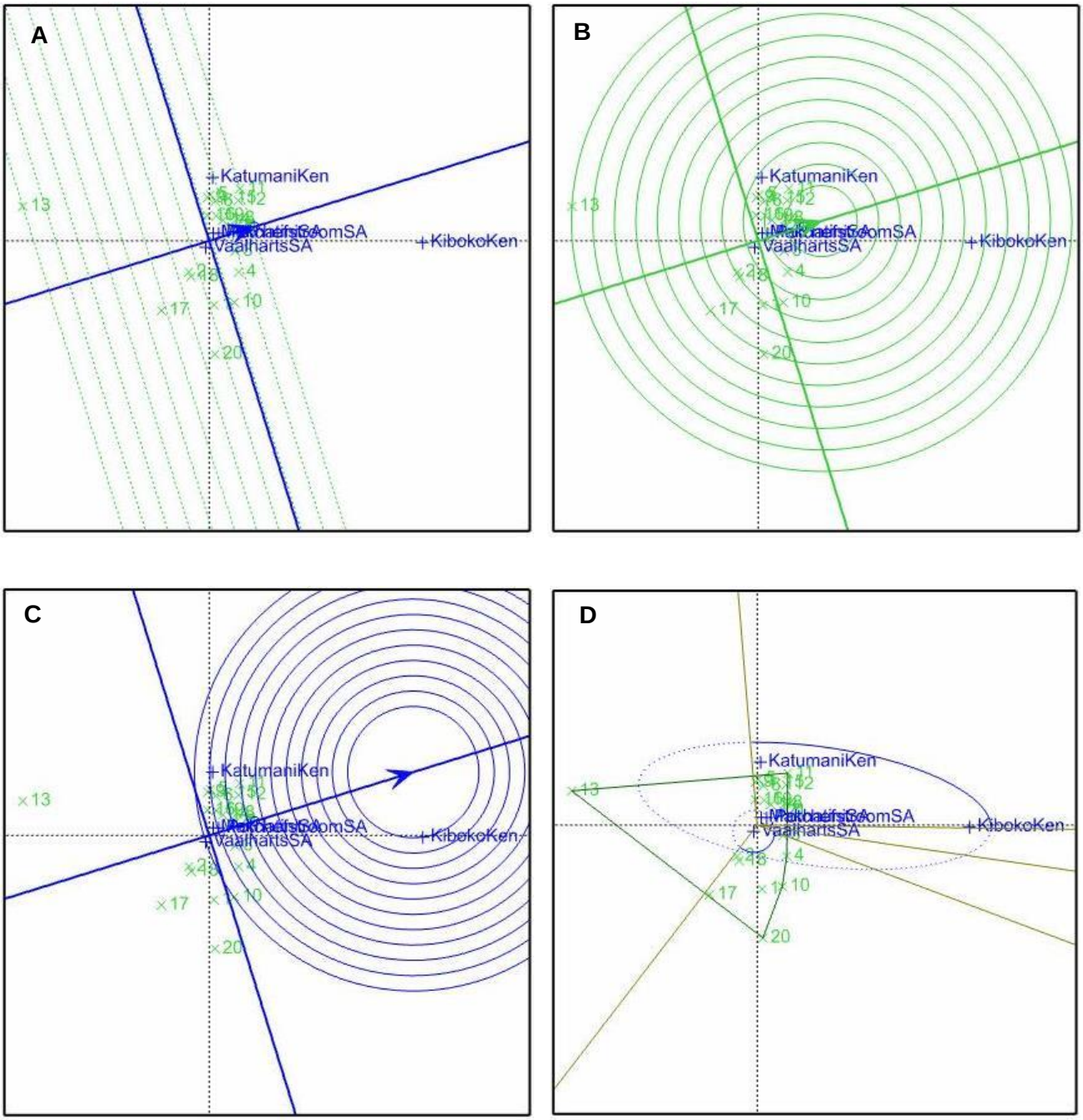

Figure 2. Genotype main effect and genotype by environment interaction biplot for *Fusarium verticillioides* colonisation in 21 maize inbred lines tested, based on environment-focused scaling for **A.** the genotype ranking, **B.** genotype comparison, **C.** environment comparison and **D.** the polygon view exhibiting mega-environments. Green and blue numbers represent genotypes and environments, respectively, with localities represented as: Potchefstroom (SA), Makhatini (SA), Kiboko (KEN) and Katumani (KEN).

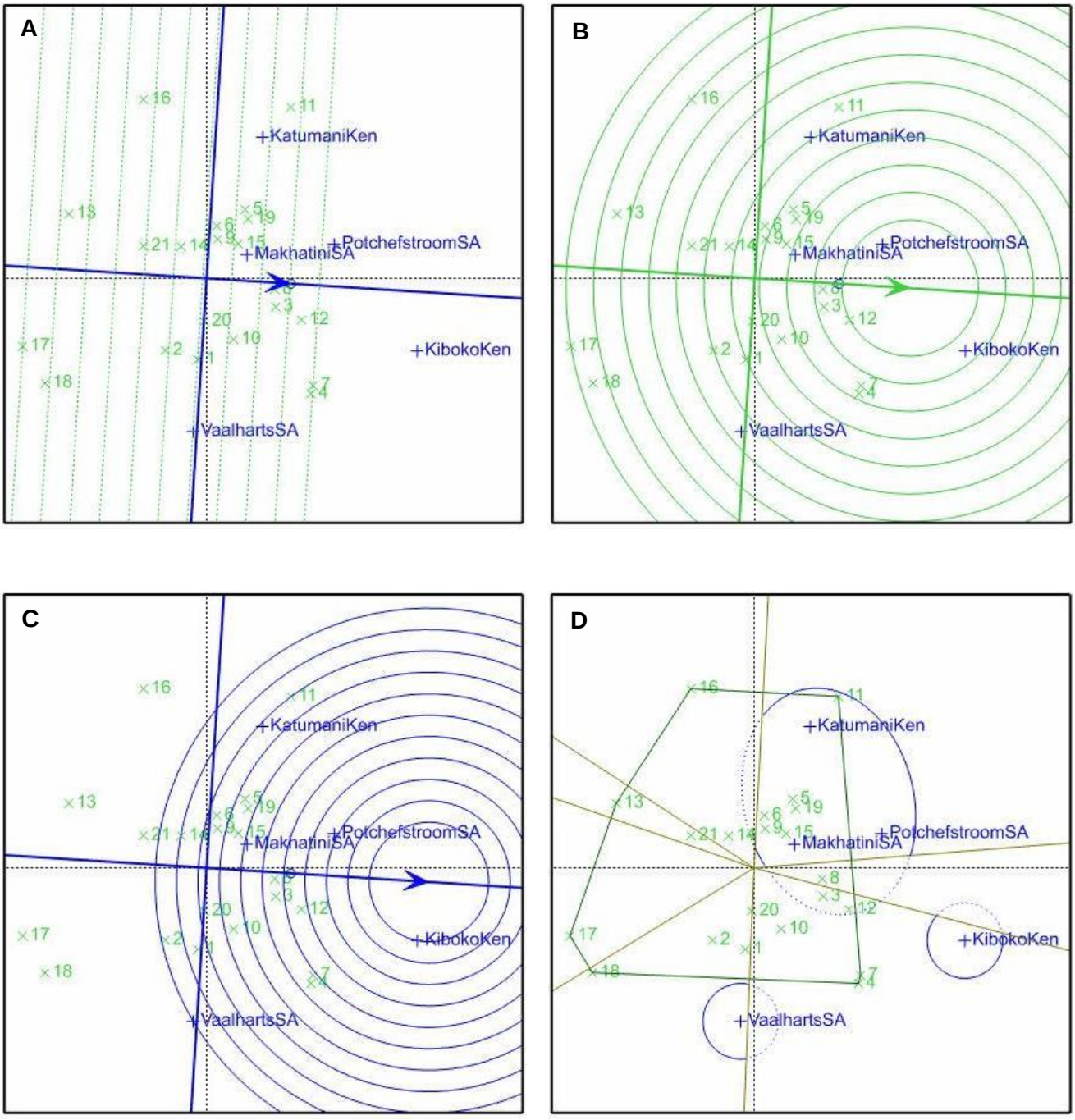

Figure 3. Genotype main effect and genotype by environment interaction biplot for total fumonisin accumulation in 21 maize inbred lines tested, based on environment-focused scaling for **A.** the genotype ranking, **B.** genotype comparison, **C.** environment comparison and **D.** the polygon view exhibiting mega-environments. Green and blue numbers represent genotypes and environments, respectively, with localities represented as: Potchefstroom (SA), Makhatini (SA), Kiboko (KEN) and Katumani (KEN).

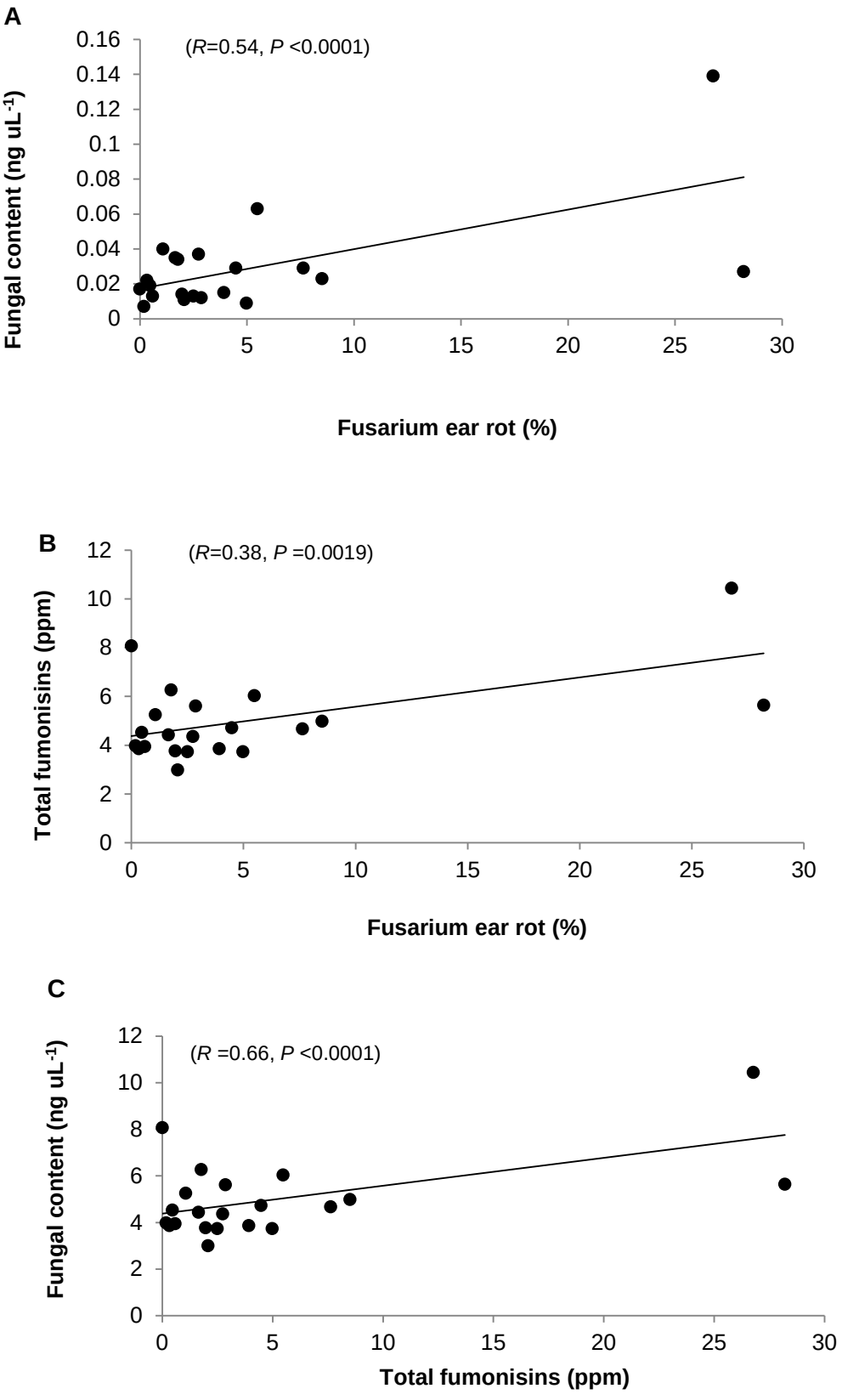

Figure 4. Pearson correlation coefficients (*R*) demonstrating the relationship between **(A)** Fusarium ear rot and fungal content, **(B)** Fusarium ear rot and total fumonisins and **(C)** total fumonisins and fungal content in maize inbred lines evaluated in Makhatini, South Africa.

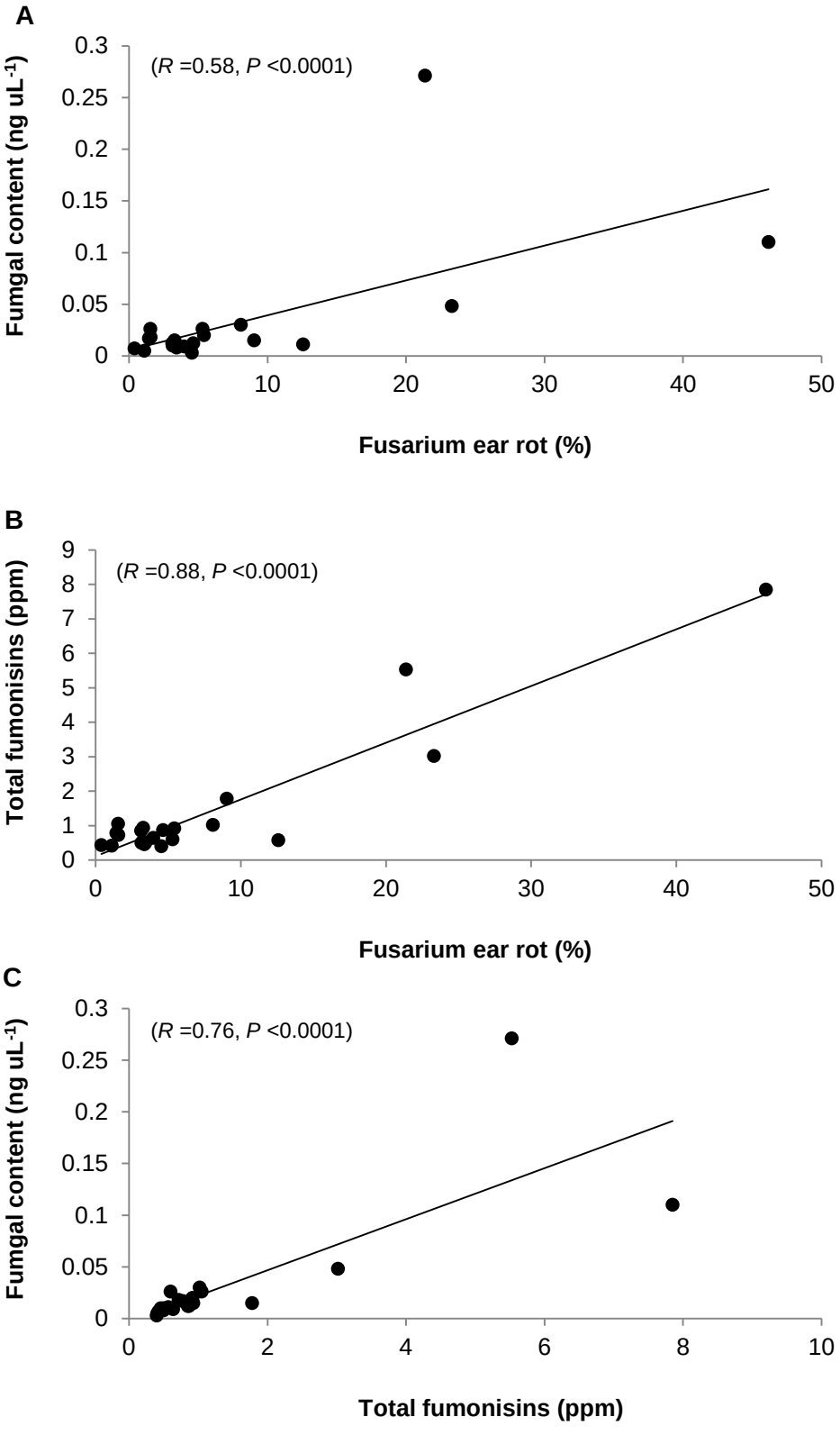

Figure 5. Pearson correlation coefficients (*R*) demonstrates the relationship between **(A)** Fusarium ear rot and fungal content, **(B)** Fusarium ear rot and total fumonisins and **(C)** total fumonisins and fungal content in maize inbred lines evaluated in Potchefstroom, South Africa.

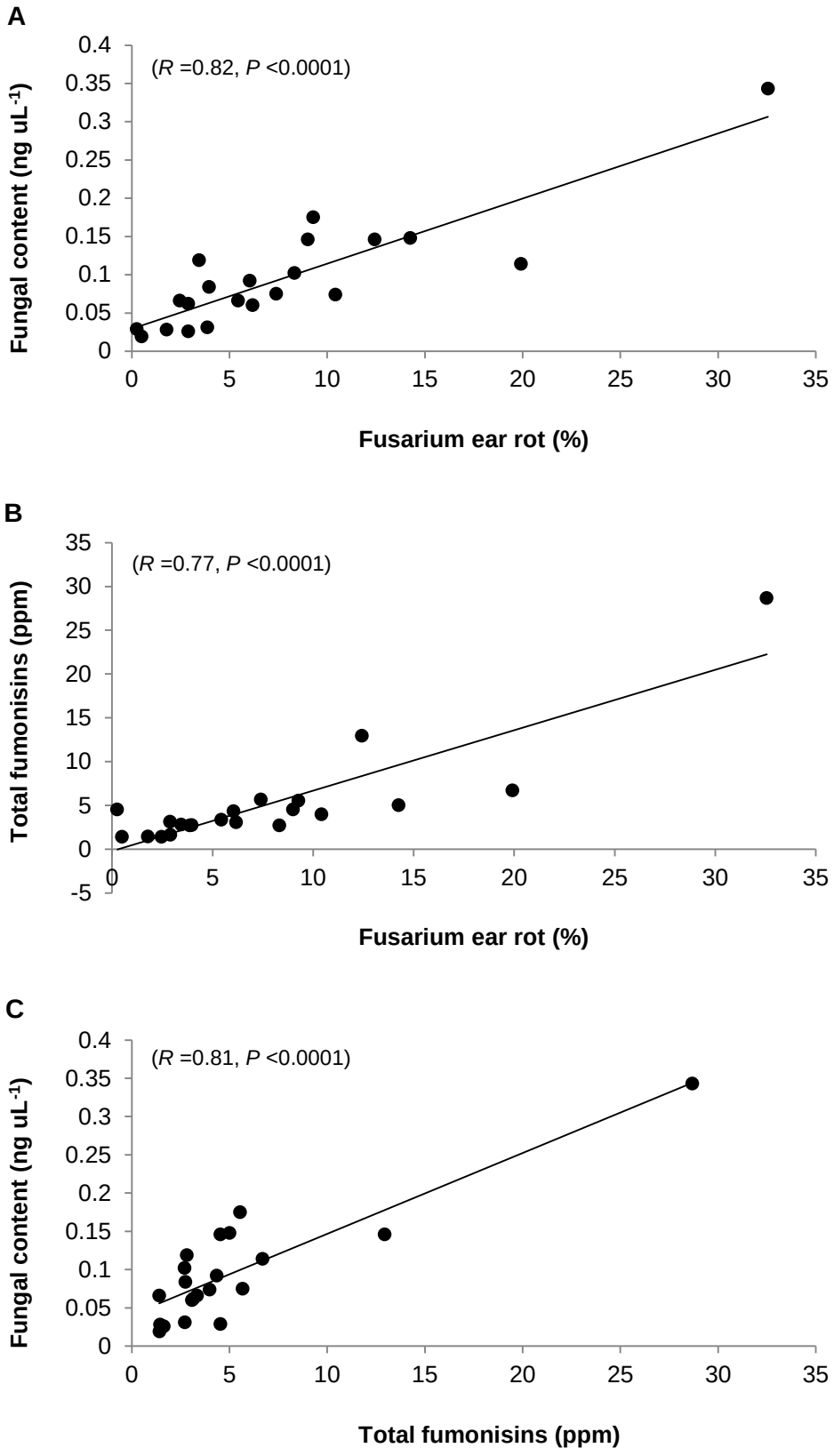

Figure 6. Pearson correlation coefficients (*R*) demonstrates the relationship between **(A)** Fusarium ear rot and fungal content, **(B)** Fusarium ear rot and total fumonisins and **(C)** total fumonisins and fungal content in maize inbred lines evaluated in Vaalharts, South Africa.

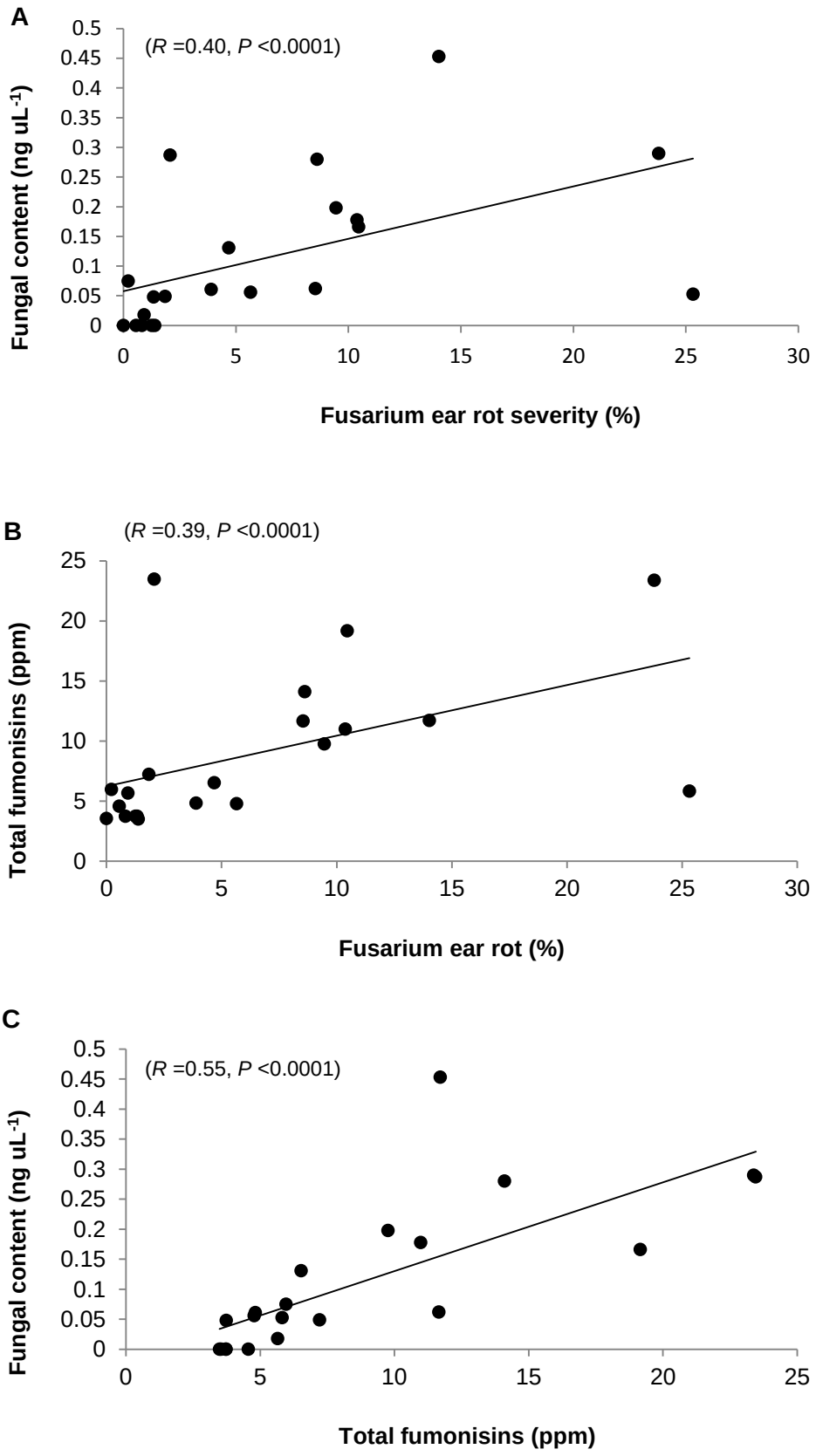

Figure 7. Pearson correlation coefficients (*R*) demonstrates the relationship between **(A)** Fusarium ear rot and fungal content, **(B)** Fusarium ear rot and total fumonisins and **(C)** total fumonisins and fungal content in maize inbred lines evaluated in Katumani, Kenya.

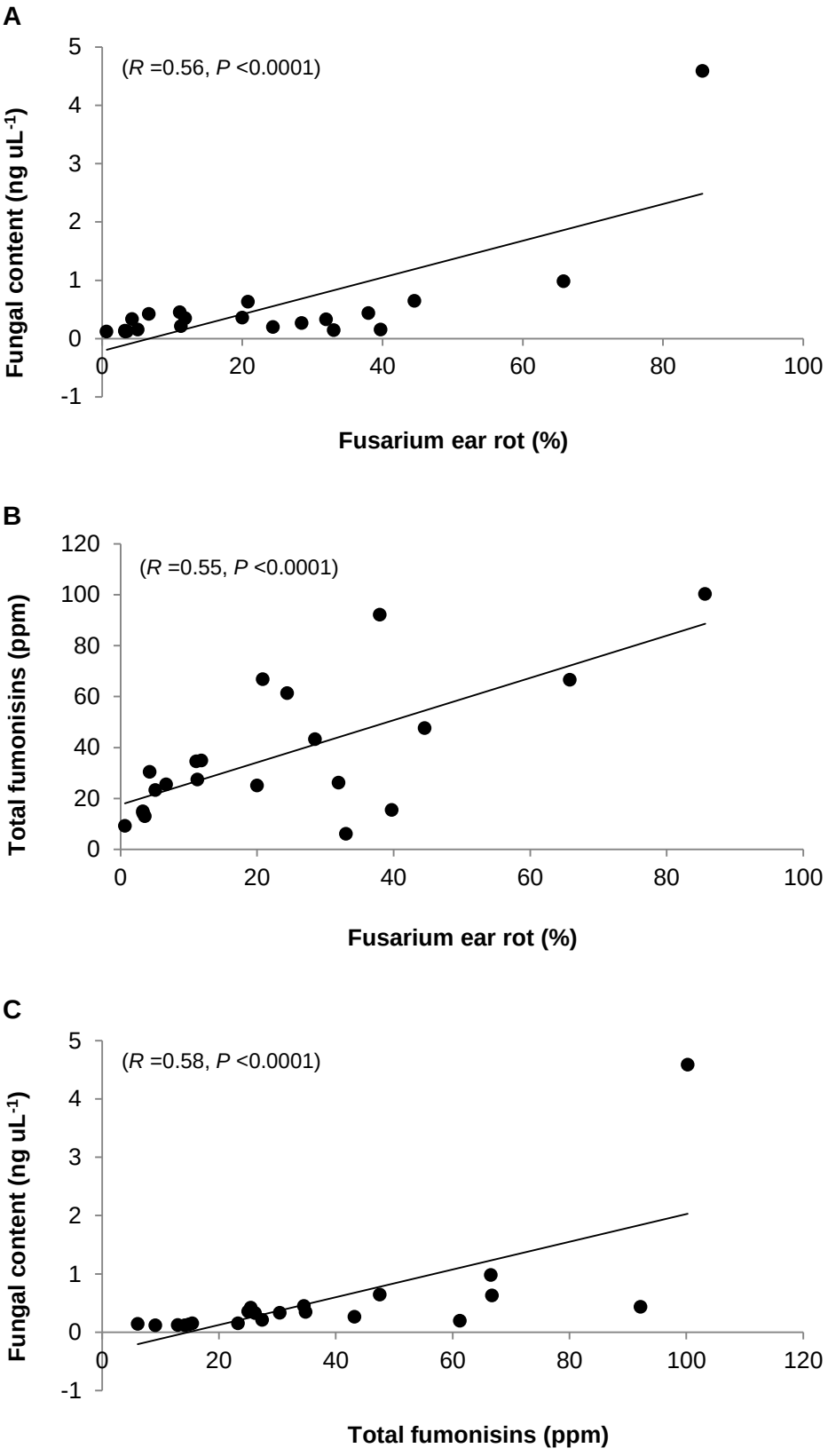

Figure 8. Pearson correlation coefficients (*R*) demonstrates the relationship between **(A)** Fusarium ear rot and fungal content, **(B)** Fusarium ear rot and total fumonisins and **(C)** total fumonisins and fungal content in maize inbred lines evaluated in Kiboko, Kenya.

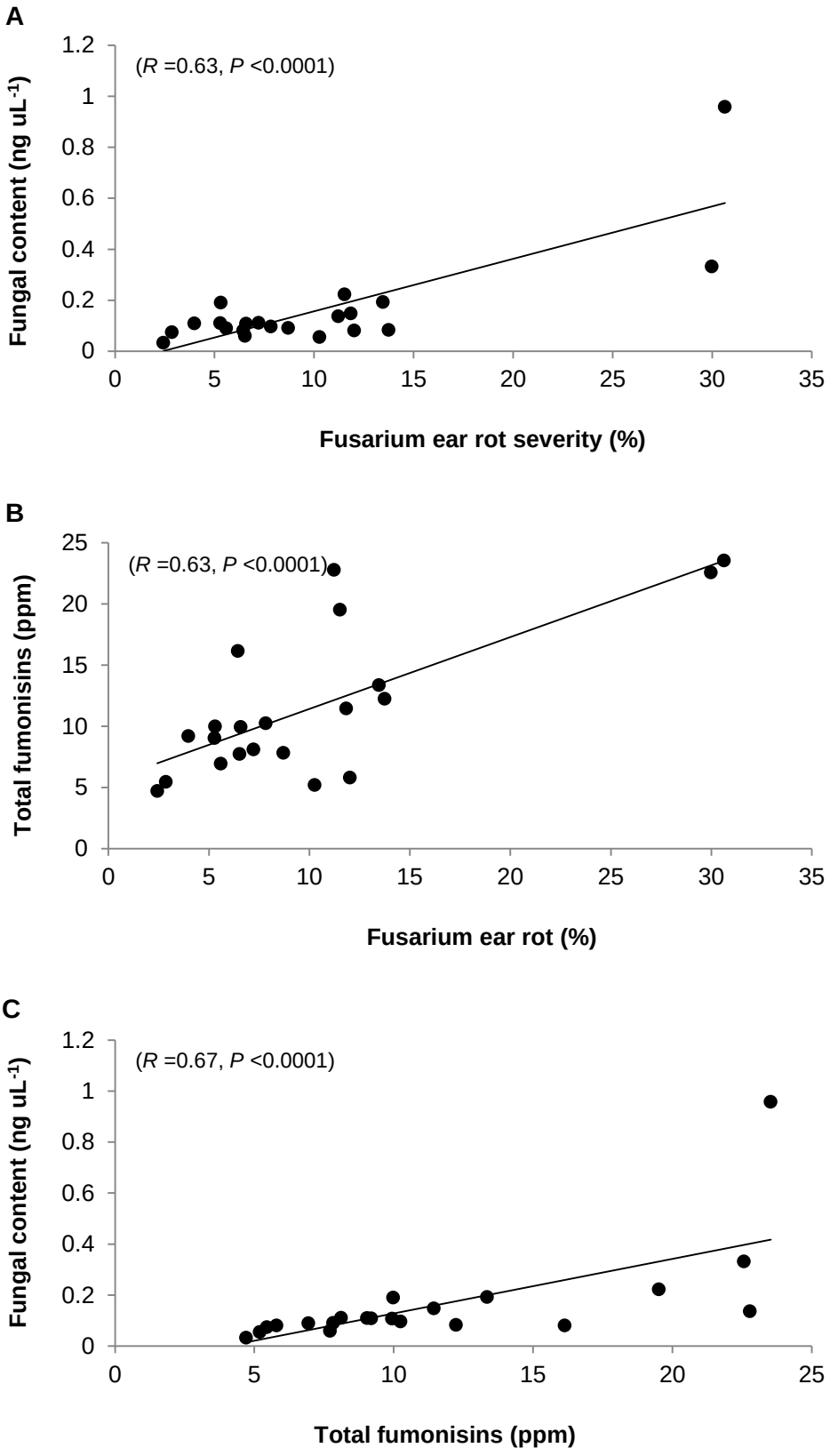

Figure 9. Pearson correlation coefficients (*R*) demonstrates the relationship between **(A)** Fusarium ear rot and fungal content, **(B)** Fusarium ear rot and total fumonisins and **(C)** total fumonisins and fungal content in maize inbred lines evaluated across all five localities.

CHAPTER 4

The use of gamma radiation to generate resistance to *Fusarium verticillioides* and fumonisin accumulation in elite maize inbred lines

ABSTRACT

Fusarium verticillioides is ubiquitously associated with maize wherever it grown. The fungus can reduce yield and grain quality, which is exacerbated by the production of toxic secondary metabolites, called fumonisins. Plant resistance is the best means to control *F. verticillioides*, but such resistance is not readily available in existing maize cultivars. In this study, gamma irradiation was employed to generate genetic variability for resistance to *F. verticillioides* and fumonisin accumulation. The irradiation load was optimised on a maize hybrid (CRN3505) and subsequently evaluated on three inbred lines I-9, I-34 and CB-248. Percentage germination, seedling survival and plant height at 7, 21 and 35 days after irradiation (dai) determined the effective radiation dosage. Plant development was also used to assess relative biological efficacy of gamma radiation. Approximately 7 000 kernels of seven elite maize inbred lines namely I-9, I-16, I-34, I-35, I-37, CB-222 and CB-248 were irradiated at a dosage of 250 Gy and field planted. Plants were annually self-pollinated and artificially inoculated with *F. verticillioides*. Plants that developed $\leq$10% FER were retained to be planted the next season and analysed for FER severity, fungal target DNA and fumonisins at the M_4 stage using visual assessment, quantitative real-time PCR and liquid chromatography tandem mass spectrometry, respectively. The seedling height of CRN3505 was significantly affected 7 and 21 dai at $\leq$150 Gy. Radio-sensitivity was displayed by lines I-34 and CB-248 at $\leq$200 Gy at 7 dai. Inbred line I-9 was not affected by gamma irradiation at 7 dai, but was significant affected 21 and 35 dai. Field testing of mutant plants over four seasons revealed that four of the 12 M_4 lines from inbred I-9, five of the 13 I-16-derived M_4 lines, six of 17 I-37- derived M_4 lines, three of 11 M_4 lines derived from CB-222 and six of 18 M_4 lines derived from CB-248 were resistant to FER and fumonisin accumulation. These lines can be used to develop *F. verticillioides*-resistant hybrids or serve as donors of resistance alleles for high yielding inbred lines. The comparison of mutated and non-mutated lines could aid in the identification and isolation of resistance genes.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important cereal crops worldwide. As a food and feed commodity, it serves as staple food for the majority of people in sub-Saharan Africa (SSA) and is used as livestock feed and raw materials for industrial products like paint, paper and adhesives (DAFF, 2014). With an expanding population approaching one billion people (World Bank, 2015), Africa requires an increase in the production of maize. Several biotic and abiotic stresses, however, significantly affect maize production on the continent. Abiotic factors include drought and climatic change (Oerke and Dehne, 2004; Barnabas *et al*., 2008, Marin *et al*., 2010), while pathogens and pests can lead to reduced yield and compromised crop value (Munkvold, 2003). South Africa is the main maize-producing country in the Southern African Developing Community (SADC) region, producing an average of 12 million tons maize annually. It also exports maize grain to neighbouring countries (GrainSA, 2015).

The pathogen most commonly associated with maize ears in South Africa is *Fusarium verticillioides* (Sacc.) Nirenberg (Boutigny *et al*., 2012). The fungus causes Fusarium ear rot (FER) and can cause seedling blight, root rot as well as stalk rot. It's presence reduces grain yield and quality due to pre-harvest contamination and discolouration of kernels that reduce its grade. *Fusarium verticillioides* also produces toxic secondary metabolites (mycotoxins), known as fumonisins, that have been associated with a number of human and animal diseases worldwide. These include oesophageal cancer in adults (Franceshi *et al*., 1990; Sydenham *et al*., 1991; Rheeder *et al*., 1992) and neural tube defects in newly born babies (Missmer *et al*., 2006). Animal diseases include leukoencephalomalacia in horses (Kellerman *et al*., 1990), liver cancer in rats (Gelderblom *et al*., 1994) and neurodegeneration in mice (Osuchowski *et al*., 2005).

The control of *F. verticillioides* infection and fumonisin deposition in maize is not easy as the fungus first colonises maize plants asymptomatically as endophytes. Management strategies include altered cultural practices such as proper fertilizer and irrigation schedules, adequate plant spacing, timely harvesting (Bush *et al*., 2004, Marin *et al*., 2010), and managing insect pests that cause wounds with pesticides and Bt maize (Munkvold *et al*., 1999). The planting of disease-resistant cultivars is considered the most effective means to control FER and fumonisin contamination of maize (Munkvold and Desjardins, 1997). Such plants provide an affordable and environmentally sound means of plant protection to both commercial and subsistence farmers. *Fusarium verticillioides*-resistant plants can be produced by conventional or unconventional breeding, such as genetic modification and mutation breeding (Duvick, 2001).

Mutation breeding has become a popular and effective tool for crop improvement (Dubinin, 1964; Acharya *et al.* 2007), and has been used to develop improved cultivars of cereals, fruits and other crops (Lee *et al*., 2002). During the past seven decades, more than 2 252 mutant varieties have been officially released (Maluszynski *et al.,* 2000), of which 320 were improved for disease and pest resistance in cereals (rice, barley, maize, wheat) and legumes (bean, green pea) (Kozjak and

Meglic, 2012). Radiation and chemical mutagenesis has been the methods most frequently used to generate diversity in existing germplasm (Mba *et al*., 2012). The prime strategy in mutation-based breeding is to alter one or two traits to improve productivity or enhance the quality of crops. Maize has been improved for increased yield and nutritional value, and for reduced plant height (Christov and Christova, 1995; Christov *et al*., 2004; Tomlekova, 2010). Mutagenesis can also be used to sterilize grains, including maize, and has been shown to break down aflatoxin B_1, produced by *Aspergillus flavus* (Link.) (Ghanem *et al*., 2008; Enu and Enu, 2014).

No maize cultivars in South Africa are resistant to FER and fumonisin contamination (Rheeder *et al*., 1990). Resistance has, however, been found in a few locally adapted breeding lines (Small *et al*.; 2012; Mouton, 2014). Introducing such resistance into local high-yielding and agronomically-adapted hybrids by conventional plant breeding would be a time-consuming and costly process. This process may be further compounded by poor general and specific combinability abilities. The current study, therefore, aimed to use irradiation to generate resistance to FER and fumonisin accumulation in South African maize inbred lines. To achieve this, a gamma irradiation protocol was optimised and maize kernels mutated and evaluated under field conditions.

MATERIALS AND METHODS

Plant material

The commercially available maize cultivar CRN3505 was initially used to determine the radio-sensitivity of maize to various ionising radiation sources, and together with inbred lines I-9, I-34 and CB-248 were used to optimize radiation dosage. Seven elite inbred lines were thereafter selected for mutation breeding (Table 1). These inbred lines were all developed for commercial purposes and have good heterotic combinability properties. They include lines resistant and susceptible to FER, *F. verticillioides* infection and fumonisin contamination (Chapter 2).

Optimisation of radiation dosage

The optimal radiation dosage required for X-ray and gamma irradiation of maize kernels was first investigated. For X-ray mutation, 50 maize kernels of the maize hybrid CRN3505 were irradiated with a LINAC X-ray (Philips SL 75-5) at the laboratories of iThemba in the Western Cape, South Africa. The dosage rate applied was 4 Gray (Gy) per minute for total radiation dosages of 90, 110, 130, 150, 170 and 190 Gy. Gamma irradiation was performed at the facility of XSIT Pty, Citrusdal, South Africa. Fifty maize kernels of the cultivar CRN3505 and inbred lines I-9, I-34 and CB-248 were subjected to a 14 kCi Co_{60} panoramic point source irradiator at a radiation dosage of 10.19 Gy/min for a total irradiation dosage of 0, 150, 200, 250, 300, 350, 400, 450 and 500 Gy, with 600 and 700 Gy additionally applied to the inbred lines only.

Germination rate and plant development were used to determine the effect of the radiation dosages. Kernels were germinated under humid conditions, and the rate of germination determined

by the number of germinating kernels in comparison to the number of kernels irradiated. After 7 days, the cotyledon was inspected for fungal infection and then transplanted into seedling trays containing sterile potting soil. The maize seedlings were allowed to grow for 2 weeks (21 days after irradiation (dai), after which the seedlings were measured from root to tip and their root architecture visually inspected for normal development. They were then transplanted into 1-L planting bags in the greenhouse. Irrigation and fertilization (1:2:1; N:P:K) of the greenhouse trials were administered manually, and maize plants were measured again 35 days after irradiation. Due to glasshouse space constraints, only a subset of plants (*n* = 15) per radiation dose were kept to determine any adverse effects of radiation on plant physiology and development, including ear development, ear and pollen synchrony and pollen viability.

Field evaluation of irradiated maize

Maize kernels of seven elite inbred lines were mass-irradiated at the facility of XSIT Pty in Citrusdal at a gamma radiation dosage of 250 Gy. One week after irradiation, 7 000 kernels of each line were planted in a field trial at the ARC-GCI, Potchefstroom (grid ref.: 26˚73'S, 27˚07'E; altitude, 1349 m), South Africa. Standard agricultural practises for the production of maize were followed. Briefly, experimental fields were sprayed with the pre-emergence herbicide flumetsum/S-metolachlor at 630 g L^{-1} (Bateleur Gold EC) and fertilised prior to planting with 3:2:1 N:P:K at a rate of 150 kg ha^{-1} + 0.5 Zn. Maize kernels were hand planted (two seeds per hill) in double-row 10-m plots, with an intra-row spacing of 0.3 m and an inter-row spacing of 1 m. All experimental plots were thinned to 33 plants per plot 3 weeks after emergence. Trials were conducted under dryland conditions and moisture stress was monitored and supplemented with overhead irrigation when required. The post-emergence herbicide halosulfuron-methyl, at 750 g kg^{-1} (Servian 75 WG/Cyprex WP), was used to manage weeds following planting. Topdressing was administered with LAN 28 using the broadcast method at the eighth leaf stage, while stalk borer infestations were controlled with the insecticide beta-cyfluthrin 0.5 g kg^{-1} (Bulldock 0.05 GR) at the 12^{th} leaf stage.

The M_0-M_4 inbred mutant ears were developed during the maize-growing seasons of 2009/10 to 2012/13. Plants were hand-pollinated and pedigree-selected. The pedigree selection method comprised the self-pollination of individual plants, the selection of an individual maize ear, and the subsequent planting of that ear using the ear-to-row method. Pollinations were performed at 50% silk emergence. After pollination the pollinating bags were secured over the pollinated maize ear for the duration of the trial. During the vegetative growth stages and at harvest, plants were visually inspected for synchrony between pollen shed and silking, low ear placement and well-filled ears. Maize ears of abnormal shape, few kernels, non-straight kernel per row and poor husk coverage were discarded. Furthermore, maize ears exhibiting >10% FER development and Diplodia ear rot (*Stenocarpella maydis* (Berk.) B. Sutton) symptoms were also discarded. Maize ears that expressed $\leq$ 10% FER were selected and hand-shelled, with those containing at least 20 kernels stored at 4°C for evaluation in the following season.

Evaluation for resistance to Fusarium ear rot and fumonisin accumulation

A highly pathogenic isolate and prolific producer of fumonisin B_1, MRC 826, was used for field inoculations (Rheeder *et al.,* 2002). A conidial suspension for artificial inoculation of maize ears was prepared according to Small *et al.* (2012). The inoculum was kept at 4^{o}C prior to and during the inoculation process, and its viability confirmed by fungal growth on PDA plates following the field inoculation. Primary maize ears of self-pollinated plants were artificially inoculated down the silk channel at the milk growth stage (Afolabi *et al.,* 2007). After harvest, disease severity was determined by estimating the percentage of each ear covered by visible disease symptoms (Clements *et al.,* 2004).

Maize ears at the M_4 generation with 10% or less FER disease symptoms were bulked per row. The kernels from each bulk were then removed, mixed well, and a 100-g kernel sample collected for fumonisin and fungal target DNA quantification. The rest of the seed was stored at 4°C for subsequent breeding purposes. Each of the 100-g samples were ground to flour using a Cyclotech sample mill (Foss Tecator, Hoganas, Sweden). Flour samples were stored at -20°C until fumonisin and fungal target DNA quantifications were performed. *Fusarium verticillioides* target DNA in maize grain was determined in a 2-g sample by quantitative real-time PCR (qPCR) according to the method described by Boutigny *et al.* (2012), and fumonisins were determined in 5-g flour samples by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described in Chapter 2.

Data Analysis

The effect of different gamma doses on maize plants 7, 21 and 35 dai was subjected to combined analysis of variance (ANOVA) using Proc GLM of SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). Associations between M_4 ears and non-irradiated inbred lines were determined and illustrated by Principle Component Analyses (PCA). The first principle component (PC1), located on the X-axis, indicates the level of resistance where inbred lines with higher PC1 values (positive or negative) are considered low risk to FER, *F. verticillioides* colonisation and fumonisin accumulation. The second principle component (PC2), located on the Y-axis, represents performance stability of genotypes where PC2 values near zero demonstrate greater adaptability of genotypes to different environments (Yan and Kang, 2003). All experiments were subjected to a test of significance at $P < 0.05$ for each trait.

RESULTS

Optimisation of irradiation dosages

X-rays irradiation had no effect on the germination and plant length of the commercial maize hybrid (CRN3505) 7 and 21 dai (data not shown). When the maize kernels were treated with gamma rays,

however, a significant reduction in cotyledon length was obtained at dosages of 350, 400 and 500 Gy. The cotyledon length of seeds treated at 450 Gy did not differ significantly from those treated with gamma doses ranging from 0-300 Gy (Fig. 1A). Seedlings from non-irradiated kernels were also significantly taller 21 dai when compared to seedlings treated with gamma radiation (Fig. 1B). The seedlings were most reduced at an irradiation dosage 500 Gy, while plant length of seeds irradiation at dosages of 350, 400 and 450 Gy did not differ significantly (Fig. 1B).

The irradiation of maize inbred lines resulted in a significant dose effect for I-34 and CB-248 (Fig. 2A). No significant differences could be determined for cotyledon length at 7 dai for inbred line I-9. The gamma doses of 200-350 Gy significantly reduced cotyledon length of I-34 irradiated kernels when compared to the non-irradiated control. The greatest length reduction was observed at 700 Gy, but not significantly more than for plants treated with 400 and 600 Gy (Fig. 2A). For inbred line CB-248, the length of non-irradiated cotyledons did not differ significantly from those irradiated with dosages ranging from 300-700 Gy. A significant reduction in cotyledons length of seeds exposed to 200 Gy was found, while a significant increase in length was observed for seed exposed to 250 Gy (Fig. 2A). Significant differences in plant length were determined for inbred line I-9 at 21 and 35 dai (Fig. 2B), but not at 7 dai. The plant lengths at all doses differed significantly from the control at 21 dai, with the exception of those exposed to 300 Gy. Plants were significantly taller at 200 Gy when compared to the non-irradiated control, while those treated with 250 and 350 were significantly shorter, but did not differ from each other. Plant lengths following gamma irradiation at 400-700 Gy were significantly shorter than the non-irradiated control (Fig. 2B).

All maize kernels treated by gamma irradiation germinated. Seedlings of both inbred lines I-9 and I-34 treated with 0–250 Gy developed normally to physiological maturity, with some effect on I-9 at a dosage of 250 Gy (Table 2). All seedlings of inbred line I-34 treated at doses of 400 Gy and higher were killed within 35 days, whereas I-9 was only affected when treated at doses of 500 Gy and above (Table 2). Treatment with 300-500 Gy increasingly affected plant development as the dose of irradiation increased. Plant aberrations at these dosages ranged from reduced plant length, low ear placement, lack of synchrony between pollen and silks and pollen sterility as determined by the lack of kernels produced following self-pollination. No seedlings exposed to 600-700 Gy survived to mature maize plants (Table 2). Considering the radio-sensitivity of inbred lines, a dosage of 250 Gy was selected to irradiate maize inbred lines for field testing.

Field evaluation of irradiated maize

In the M_0 plants between 0.97 and 3.6% of the self-pollinated mutants produced M_1 ears for the next generation (Table 3). The bulk of M_0 plants could not be self-pollinated due to the high number of plants, which did not produce kernels following pollination, or were disposed of because of morphological aberrations observed in the field. These included tassel seed phenotype, rough sheath characterised by failure of the leaves to open (uncurl), mutations in chlorophyll resulting in a distinct yellow colouration across the leaf blade, reduced stalk integrity resulting in the bending over

of plants, reduced plant length, inappropriate ear placement, poor synchrony of pollen and maize silks and poor pollen viability as determined by kernels obtained from self-pollination (Fig. 3). Of the M_1 plants visually assessed for FER severity during the 2010/11 season, between 23.7 (I-37) and 71.6% (CB-248) showed ≤10% FER symptoms of M_2 ears (Table 3 and 4A). Disease development during this season was good, with FER severity ranging from 0-100% (Table 4A). FER development during 2011/12 in M_3 ears also allowed for discrimination between highly infected and resistant maize ears, with some ears being fully covered by the disease. The number of ears showing ≤10% FER symptoms increased to more than 79.8% (I-9) (Table 3 and 4A).

Evaluation of M_4 mutants for resistance to Fusarium ear rot and fumonisin accumulation

FER symptoms on M_4 ears ranged from 0-52% (Table 4B). Inbred line I-9 had the least number of plants showing ≤5 (33.3%) and ≤10 (27.1%) % FER severity, while 52.0 and 26.0% of the M_4 ears derived from I-16 showed ≤5 and ≤10% FER, respectively (Table 3 and 4B). The percentage M_4 ears from I-34 that had ≤5 and ≤10% FER was 50 and 25.0%, respectively, whereas no M_4 ears from inbred line I-35 showed less than 5 or 10% FER severity (Table 3 and 4B). FER symptoms on inbred line I-37 ranged from 0–50% on M_4 ears, with 69.9% of these expressing less than 5% FER severity. Inbred lines CB 222 (53.2%) and CB 248-derived M_4 ears (64.4%) showed ≤5% FER while 23.4 and 20.0% of the harvested ears displayed ≤10% FER (Table 4B).

Three entries (170, 181 and 182), derived from inbred I-9, contained very low levels of *F. verticillioides* (0.019, 0.012 and 0.001 ng uL^{-1}, respectively) and fumonisins (0.27, 0.02 and 0.00 mg kg^{-1}, respectively) (Table 4). Entries 194, 199, 201 and 204 showed FER severity between 6.5 and 24.6% but contained low levels of *F. verticillioides* (0.001-0.011 ng uL^{-1}) and fumonisins (0.00-0.01 mg kg^{-1}). Line I-16-derived entries 135, 138, 143 and 149 displayed less than 5% FER, with *F. verticillioides* target DNA of between 0.001 and 0.042 ng uL^{-1} and fumonisin levels ranging from 0.00-0.06 mg kg^{-1}. Entry 38 of inbred I-34 had no FER symptoms, and contained 0.001 ng uL^{-1} fungal DNA and 0.92 mg kg^{-1} fumonisins. Both entries of I-35 displayed more than 5% FER and contained fumonisin levels of more than 2 mg kg^{-1}. Six of 19 entries from I-37 had ≤5% FER (41, 42, 47, 62, 63, 69 and 86) and also contained low levels of *F. verticillioides* (0.003-0.037 ng uL^{-1}) and fumonisins (0.92-1.02 mg kg^{-1}). Entries 223, 227, 235, 253 and 262, derived from CB-222, all showed ≤5% FER with fungal concentrations that ranged from 0.001-0.006 ng uL^{-1} and fumonisin content that ranged from 0.00-0.04 mg kg^{-1}. Eleven of 19 entries obtained from inbred line CB-248 displayed ≤5% FER, ≤0.020 ng uL^{-1} and ≤0.16 mg kg^{-1} fumonisins.

Assessment of FER severity, *F. verticillioides* content and total fumonisins of M_4 selections revealed that the first two principal components accounted for 60.45 (F1) and 30.25% (F2) of the variation, representing 90.7% of the total variation (Fig. 4). Fungal content and total fumonisins accounted for the most variation represented by F1 (60.45%), while FER accounted for the most variation represented by F2 (30.25%). No correlation between FER severity and fungal content (R = 0.14) was observed, and a significant though weak correlation between FER severity and total

fumonisins (R = 0.27) was obtained (P < 0.05) (Table 6). The fungal content and total fumonisin accumulation in the grain of M_4 selections correlated well (R = 0.71). The nine non-irradiated controls, as well as 24 of the 73 M_4 selections in quadrant II and III of the biplot, were more contaminated with fungal tissue and fumonisin compared to selections present in quadrant I and IV (Fig. 4). Furthermore, non-irradiated lines and selections in quadrant III were less affected by FER compared to the non-irradiated lines and selections in quadrant II. The best performing M_4 selections for all the parameters were present in quadrant IV. Most of these selections were derived from the inbred line CB-248 (Fig. 4).

Of the 12 M_4 selections derived from line I-9, 170, 181, 182 and 204 (quadrant IV) were most resistant to FER, fungal colonisation and fumonisin accumulation (Fig. 5). Selections 169, 180, 194, 199 and 201 (quadrant I) were more susceptible to FER, but contained low levels of fungal target DNA and fumonisins. Selection 177 and the non-irradiated control was susceptible to all parameters, while selections 184 and 188 were more contaminated with *F. verticillioides* and fumonisins (Fig. 5). Principle components accounted for 94.8% of the total variation, with fungal content and total fumonisins (F1) accounting for 61.6% of the variation, and FER (F2) for 33.2% of the variation (Fig. 5). The percentage FER neither correlated with fungal content nor with total fumonisins, while fungal content and total fumonisins correlated very well (R = 0.84) (Table 6).

The M_4 selections derived from I-16 accounted for 94.9% of the variation, with F1 (fungal content and total fumonisins) and F2 (FER) representing 58.5 and 36.4% of the variation, respectively (Fig. 6). Three selections (135, 138 and 107B) were the best performing selections for FER, *F. verticillioides* colonisation and fumonisin accumulation. Selections 108, 143, 144, 149 and 152 also contained low levels of fungal target DNA and fumonisins, but were more affected by FER. Selection 107A, along with the non-irradiated controls, was the most susceptible to all three parameters. The selections 116, 133, 137 and 148 showed low FER severity but contained more fungal target DNA and fumonisins than selections in quadrant I and IV (Fig. 6; Table 5). Seven selections derived from line I-37 (47, 64, 68, 69, 80, 92 and 93) accumulated less FER, fungal target DNA and fumonisins compared to the 10 other M_4 selections and the non-irradiated controls (Fig. 7). Non-irradiated controls associated with selections 58 and 75 in quadrant III, characterised by higher FER severity, fungal target DNA and fumonisins in comparison to selections in quadrant I and IV (Fig. 7; Table 5). Principle components accounted for 79.25% of the total variation, ascribing 56.01% to F1 and 23.24% to F2. All the parameters were responsible for the variation represented by F1 and F2. No significant relationship between any of the variables were determined (Table 6).

Selections from inbred line CB-222 that were most resistant to FER, fungal and fumonisin contamination grouped in quadrant IV. Those selections, that were more susceptible to FER but still resistant to *F. verticillioides* and fumonisin accumulation, grouped in quadrant I (Fig. 8). The non-irradiated controls, together with selection 237 (quadrant III) developed less than 10% FER but contained high levels of fungal material and fumonisin. Selections 259 and 218 (quadrant IV) were most affected by FER and contained high levels of fungal target DNA and fumonisins (Fig. 8; Table

5). Only fungal content and fumonisin accumulation had a strong, positive correlation (*R* = 0.87), and all the parameters were responsible for the variation represented by F1 and F2.

Eight of the 18 M_4 selections derived from inbred line CB-248 (quadrant IV) were resistant to *F. verticillioides* and fumonisin accumulation (Fig. 9). The non-irradiated control and selections (321) and 312 were most affected by FER, severity differed in their response to fungal and fumonisin contamination. Selections 269, 272, 277, 280, 296 and 314 (quadrant I) developed high levels of FER but contained less fungal target DNA and fumonisins than selections in quadrant II and III (Fig. 9; Table 5). The variation represented by F1 (69.9%) was predominantly the result of fungal and fumonisin contamination of maize grain, while F2 (25.3%) was as the result of FER (Fig. 9). Percentage FER correlated significantly moderate with total fumonisins (*R* = 0.49), while fungal content and fumonisin contamination had a strong positive relationship (*R* = 0.82) (Table 6).

DISCUSSION

One of the main challenges in improving crops for disease resistance is finding sources of resistance. Such sources can be available in existing cultivars or breeding materials, or in wild relatives of agricultural crops (Polák and Barton, 2002). Once discovered, resistance needs to be introduced into cultivated crops, either through conventional breeding or genetic modification. Maize breeding and genetic modification, however, may have some limitations. The breeding of new maize cultivars is expensive, labour intensive, and can take between 10 and 12 years (De La Fuente *et al*., 2013), while the production of genetically modified foods are not allowed in most countries. Mutation breeding, if successful, provides an important alternative to overcome these limitations. In this study, gamma irradiation was investigated for the first time as a means to generate genetic diversity for resistance in maize to *F. verticillioides* and fumonisin accumulation. With the exception of one inbred line (M-35), M_4 mutants from all the inbred lines irradiated demonstrated improved resistance to FER, *F. verticillioides* colonisation and fumonisin accumulation when compared to their non-irradiated elite lines.

The method and dosage used for ionizing radiation is essential for effective mutation breeding of maize. In this study, X-ray mutation appeared to be ineffective in generating variation. Gamma irradiation, however, had a significant effect on germinated seeds, seedlings and mature plants. The greater penetrating ability of gamma rays may have resulted in more mutations compared to high-energy X-rays (Moussa, 2006). Gamma rays have also proved to be an economical and effective means to mutate other agricultural crops, including *Eruca vescaria* subsp. *sativa* (L.) (Moussa, 2006), *Musa* spp. (Kulkarni *et al*., 2007), *Triticum* spp. (Melki and Marouani, 2009; Singh and Datta, 2010) and *Vigna unguiculata* (L.) (de Ronde and Spreeth, 2007). Other ionising radiations have been shown to induce genetic variability in maize (Stadler and Roman, 1948; Conger, 1976). Chemical mutagens such as ethyl methane sulfonate (EMS) have also been used to induce genetic variation in maize (Till *et al*., 2004; Kumar and Kumar Rai, 2009) and *Musa*

(Bhagwat and Duncan, 1998). Nonetheless, gamma and X-rays are the most commonly used physical mutagens (Mba *et al*., 2012). Gamma radiation has also been used to produce disease resistance in *Helianthus annuus* L. to Alternaria leaf spot (de Oliviera *et al*., 2004).

Seed-irradiation experiments with maize inbreds and hybrids have shown that hybrids were more resistant to sparsely and densely ionizing radiations than inbreds (Conger, 1976). No attempt, however, was made to relate the response to nuclear variables, and no values for relative biological effectiveness (RBE) were given. When inbred lines and hybrids were exposed to low doses of gamma irradiation, the hybrids produced seedlings with increased root and shoot lengths (Marcu *et al*., 2014). This has important implications for current research, as future irradiation efforts should involve irradiation of high-yielding and locally-adapted maize hybrids for FER and fumonisin resistance. Such mutant hybrids can then immediately be utilised, after a selection process, instead of incorporating the mutations from inbred lines into hybrids by conventional breeding.

The development of maize seedlings and plants were affected when exposed to increasing levels of gamma radiation. The germination rate of kernels was not affected by any dosage up to 700 Gy. Normal plant development, however, was only observed when kernels were treated with <250 Gy. This is consistent with earlier studies that showed that the growth and development of wheat was affected by 100-400 Gy doses of gamma radiation (Mashev *et. al*., 1995; Irfaq and Nawab, 2001; Marcu *et al*., 2013). Seedling mortality increased as irradiation dose increased with little to no seedling survival when seeds were exposed to <500 Gy. One possible explanation is that chromosomal damage affected survival, similar to the physiological response of *Orthosiphon stamineus* (Benth.) (Chinese medicinal herb) following gamma irradiation (Kiong *et al*., 2008). Similar findings were obtained by Marcu *et al*. (2013) following gamma irradiation of maize seeds with <500 Gy while gamma doses of <400 Gy decreased plant survival in three wheat cultivars (Irfaq and Nawab, 2001). Growth inhibition due to high radiation doses has been attributed to cell cycle arrest in the G2/M phase during somatic cell division as well as to a variety of damages in the entire genome (Preuss and Britt, 2003).

The dosage selected for irradiation of maize inbred lines (250 Gy), which was well below that found to be radiosensitive to cotyledons and seedlings, still had a significant effect on plants grown in the field. This dosage was higher than the radiation dosage that had been used for mutation breeding of maize (Lysikov, 1999), sorghum (Larik *et al*., 2009) and sunflowers (de Oliviera *et al*., 2004). Although most of the M_0 plants developed normally, the range of off-types developed through mutation breeding in this study demonstrated the value of the technology beyond disease resistance. Gamma irradiation could potentially be used for generating smaller and taller maize plants, increased yield, shorter growth cycles and improved nutritional value. The artificial inoculation with *F. verticillioides* and the selection for visibly reduced FER symptoms at each inbreeding stage resulted in the selection of M_4 lines with improved resistance to FER, *F. verticillioides* colonisation and fumonisin accumulation. Such a selection, however, can compromise the yield potential and combinability of elite maize inbred lines. In this study, plant selection for

desirable agronomic traits occurred concurrently with screening for resistance to ensure that resistant selections were also agronomically acceptable. Several mutant lines were resistant to FER, but contained high levels of fungal target DNA and fumonisins. Other lines were susceptible to FER but resistant to fungal and fumonisin contamination. Such disparities between disease expression and fumonisin content is not uncommon (Clements *et al*., 2004; Afolabi *et al*., 2007, Small *et al*., 2012; Chapters 2 and 3) and underscores the importance of testing for fumonisin content of maize grain to ensure resistance to *F. verticillioides*.

The mutant maize inbred lines developed in this study need further testing before they can be employed for resistance breeding to *F. verticillioides* and reduced fumonisins in South Africa. Irradiation may have affected the yield potential and combining abilities of the M_4 selections while additionally creating genetic variation for traits such as drought tolerance and resistance to other ear rot pathogens. Maize yield has in the past been increased following treatment with doses of up to 250 Gy, with the optimum yield occurring at 150 Gy (Mokobia *et al*., 2006). The yield, stability and combinability of *F. verticillioides*-resistant M_4 selections should, therefore, be determined. The comparison of mutated and non-mutated lines using advanced genomic and proteomic studies could further aid in the isolation and identification of genes for resistance to *F. verticillioides* and reduced fumonisins. This would allow resistance alleles to be incorporated into other high-yielding but *F. verticillioides*-susceptible lines.

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Table 1. Maize inbred lines selected for mutation breeding.

Line Number	Inbred line	Genetic background	Origin[1]	FER/fumonisin[2]	Kernel Colour
1	I-9	Iodent	ARC-GCI- South Africa	Susceptible	White
2	I-16	Iodent	ARC-GCI- South Africa	Susceptible	White
3	I-34	Iodent	ARC-GCI- South Africa	Susceptible	White
4	I-35	Iodent	ARC-GCI- South Africa	Susceptible	White
5	I-37	Iodent	ARC-GCI- South Africa	Intermediate	White
6	CB-222	Corn belt	ARC-GCI- South Africa	Intermediately Resistant	White
7	CB-248	Corn belt	ARC-GCI- South Africa	Intermediately Resistant	White

[1]Agricultural Research Council – Grain Crops Institute (ARC-GCI)
[2]Refer to Chapter 2

Table 2. Germination rate and plant development following irradiation of dry maize kernels of inbred lines I-9 and I-34.

Gamma dose (Gy)	Inbred line	# Seeds	Germination (%)	Seedling survival 21 dai (%)	Seedling survival 35 dai (%)	Plant development
0	**I-9**	50	100	94.0	94.0	Normal
	I-34	50	100	98.0	92.0	Normal
200	**I-9**	50	100	92.0	90.0	Normal
	I-34	50	100	82.0	80.0	Normal
250	**I-9**	50	100	90.0	88.0	Chlorophyll, tassel seed, normal
	I-34	50	100	100.0	86.0	Normal
300	**I-9**	50	100	94.0	82.0	Reduced length, chlorophyll
	I-34	50	100	68.0	66.0	Reduced length, tassel seed and chlorophyll
350	**I-9**	50	100	98.0	86.0	Reduced length, tassel seed, chlorophyll
	I-34	50	100	68.0	66.0	Tassel seed, chlorophyll, no kernels following pollination
400	**I-9**	50	100	94.0	88.0	Abnormal leaf formation, reduced length, no kernels following pollination
	I-34	50	100	0.0	0.0	No plants survived to adult-stage
500	**I-9**	50	100	84.0	22.0	Abnormal leaf formation, poor synchrony, no kernels following pollination
	I-34	50	100	0.0	0.0	No plants survived to adult-stage
600	**I-9**	50	100	60.0	0.0	No plants survived to adult-stage
	I-34	50	100	0.0	0.0	No plants survived to adult-stage
700	**I-9**	50	100	18.0	0.0	No plants survived to adult-stage
	I-34	50	100	0.0	0.0	No plants survived to adult-stage

Table 3. Number of maize ears with less than 10% Fusarium ear rot (FER) of the M_1 and M_2 generations and less than 5% FER of the M_3 generations.

	Inbred lines							
	I-9	***I-16***	***I-34***	***I-35***	***I-37***	***CB-222***	***CB-248***	***Total***
M_0 kernels irradiated 2009/10	7 000	7 000	7 000	7 000	7 000	7 000	7 000	49 000
M_1 ears planted 2010/11	184	253	82	138	200	68	235	1 160
M_2 ears visually assessed	303	322	59	210	215	114	342	1 565
M_2 ears $\leq$10% FER	135	103	20	129	51	78	245	761
	44.6%	**32.0%**	**33.9%**	**61.4%**	**23.7%**	**68.4%**	**71.6%**	
M_2 ears replanted 2011/12	129	101	18	8	47	78	244	625
M_3 ears visually assessed	208	186	50	19	47	67	714	1 291
M_3 ears $\leq$10% FER	166	156	43	19	45	63	688	1 180
	79.8%	**83.9%**	**86.0%**	**100.0%**	**95.7%**	**94.0%**	**96.4%**	
M_3 ears replanted 2012/13	50	50	23	13	45	48	50	279
M_4 rows bulked for analysis	48	50	4	2	45	47	45	241
M_4 ears $\leq$5% FER	16	26	2	0	31	25	29	129
	33.3%	**52.0%**	**50.0%**	**0.0%**	**69.9%**	**53.2%**	**64.4%**	

*The seed of maize ears were planted ear-to-row, with a single ear per row selected for the next breeding generation

Table 4. Fusarium ear rot severity of **A)** M_2, M_3 and **B)** M_4 maize ears during the 2010/11, 2011/12 and 2012/13 maize growing seasons.

A)

	0-10%		11-25%		26-50%		51-75%		76-100%	
Inbred name	***2010/11***	***2011/12***	***2010/11***	***2011/12***	***2010/11***	***2011/12***	***2010/11***	***2011/12***	***2010/11***	***2011/12***
I-9	44.6	79.8	18.8	5.8	14.5	4.3	8.9	3.4	12.9	6.7
I-16	32.0	83.9	21.6	8.6	14.2	4.8	4.9	0.5	8.3	2.2
I-34	35.6	86.0	16.9	4.0	18.6	0.0	11.9	2.0	18.6	8.0
I-35	61.0	100.0	13.3	0.0	10.5	0.0	3.8	0.0	10.0	0.0
I-37	25.1	95.7	16.7	4.3	15.8	0.0	9.8	0.0	33.5	0.0
CB-222	71.1	94.0	13.2	4.5	7.0	1.5	2.6	0.0	6.1	0.0
CB-248	71.8	96.4	14.4	2.0	5.0	0.0	0.9	0.3	5.0	1.3

B)

Inbred name	**0-5%**	**6-10%**	**11-20%**	**21-50%**	**50-75%**	**76-100%**
I-9	33.3	27.1	25	14.6	0.0	0.0
I-16	52.0	26.0	16.0	6.0	0.0	0.0
I-34	50.0	25.0	25.0	0.0	0.0	0.0
I-35	0.0	0.0	50.0	50.0	0.0	0.0
I-37	69.9	23.1	15.4	1.9	0.0	0.0
CB-222	53.2	23.4	17.0	4.3	2.1	0.0
CB-248	64.4	20.0	13.3	2.2	0.0	0.0

Table 5. Mean percentage Fusarium ear rot severity (FER), *F. verticillioides* colonisation and total fumonisin content maize M_4 ears during the 2012/13 maize growing season at Potchefstroom.

Original inbred	Entry #	Plot number	FER (%)	Fungal content (ng uL^{-1})	Total fumonisins (mg kg^{-1})
I-9	1	169	27.7	0.023	0.18
	2	170	2.6	0.019	0.27
	3	177	14.6	0.042	0.36
	4	180	11.2	0.002	0.00
	5	181	0.2	0.012	0.02
	6	182	3.2	0.001	0.00
	7	184	8.6	0.189	2.64
	8	188	9.2	0.185	1.86
	9	194	24.5	0.011	0.01
	10	199	24.6	0.003	0.00
	11	201	16.9	0.003	0.00
	12	204	6.5	0.001	0.00
	13	215_NR*	10.0	0.130	9.90
I-16	14	107A	8.8	0.066	7.25
	15	107B	3.1	0.039	1.79
	16	108	17.0	0.003	1.58
	17	116	0.0	0.106	1.78
	18	133	4.4	0.099	0.93
	19	135	0.0	0.025	0.05
	20	137	1.5	0.232	2.82
	21	138	0.0	0.042	0.06
	22	143	4.4	0.001	0.00
	23	144	11.5	0.001	0.00
	24	148	6.1	0.352	6.95
	25	149	2.7	0.001	0.00
	26	152	9.8	0.055	0.43
	27	158_NR*	8.5	0.135	7.19
	28	160_NR*	7.2	0.107	6.95
I-34	29	38	0.0	0.001	0.92
	30	40	10.2	0.118	5.70
I-35	31	12	12.8	0.006	2.66
	32	16_NR*	17.4	0.180	14.12
I-37	33	41	0.0	0.020	1.01
	34	42	0.0	0.003	0.93
	35	47	3.7	0.004	0.94
	36	58	13.3	0.015	0.94
	37	62	1.3	0.037	1.02
	38	63	1.5	0.007	0.92
	39	64	2.1	0.002	3.67
	40	67	1.5	0.013	1.45
	41	68	7.9	0.003	1.00
	42	69	3.9	0.003	1.01

Original inbred	Entry #	Plot number	FER (%)	Fungal content (ng uL^{-1})	Total fumonisins (mg kg^{-1})
I-37	43	75	28.5	0.009	0.93
	44	80	1.2	0.007	2.72
	45	86	1.3	0.029	0.94
	46	90	13.5	0.055	0.92
	47	92	6.5	0.004	0.93
	48	93	10.3	0.002	0.93
	49	95	6.4	0.048	0.94
	50	104_NR*	27.6	0.048	7.47
	51	105_NR*	16.9	0.044	6.46
CB-222	52	218	12.9	0.027	0.93
	53	222	15.5	0.002	0.01
	54	223	1.8	0.001	0.00
	55	227	2.5	0.002	0.00
	56	235	2.0	0.003	0.01
	57	237	6.0	0.019	0.13
	58	253	0.3	0.006	0.00
	59	254	8.1	0.002	0.00
	60	259	14.1	0.028	0.31
	61	261	6.5	0.007	0.00
	62	262	1.4	0.005	0.04
	63	265_NR*	8.8	0.031	2.29
	64	266_NR*	9.0	0.031	2.43
CB-248	65	269	3.4	0.004	0.00
	66	272	8.5	0.001	0.03
	67	274	0.0	0.020	0.00
	68	277	13.1	0.003	0.00
	69	280	9.5	0.004	0.02
	70	283	0.0	0.015	0.16
	71	286	5.8	0.037	0.32
	72	290	0.6	0.007	0.00
	73	293	0.7	0.002	0.00
	74	294	0.2	0.013	0.04
	75	296	1.5	0.003	0.00
	76	298	0.0	0.003	0.00
	77	299	0.5	0.009	0.01
	78	302	1.0	0.007	0.07
	79	304	6.2	0.029	1.37
	80	307	9.0	0.055	0.62
	81	312	25.0	0.004	0.00
	82	314	1.0	0.002	0.00
	83	321_NR*	25.2	0.062	4.61
Mean			***7.6***	***0.036***	***1.5***

*Non-irradiated inbred lines

Table 6. Correlations between Fusarium ear rot severity, *F. verticillioides* colonisation and total fumonisins in M_4 selections.

Line	FER vs Fungal content	FER vs Fumonisins	Fungal content vs Fumonisins
I-9	-0.03	-0.05	**0.84***
I-16	-0.13	0.30	**0.73***
I-34	N/A	N/A	N/A
I-35	N/A	N/A	N/A
I-37	0.31	0.38	0.33
CB-222	0.52	0.47	**0.87***
CB-248	0.28	**0.49***	**0.82***
Combined	0.14	**0.27***	**0.71***

*Significant (P<0.05)

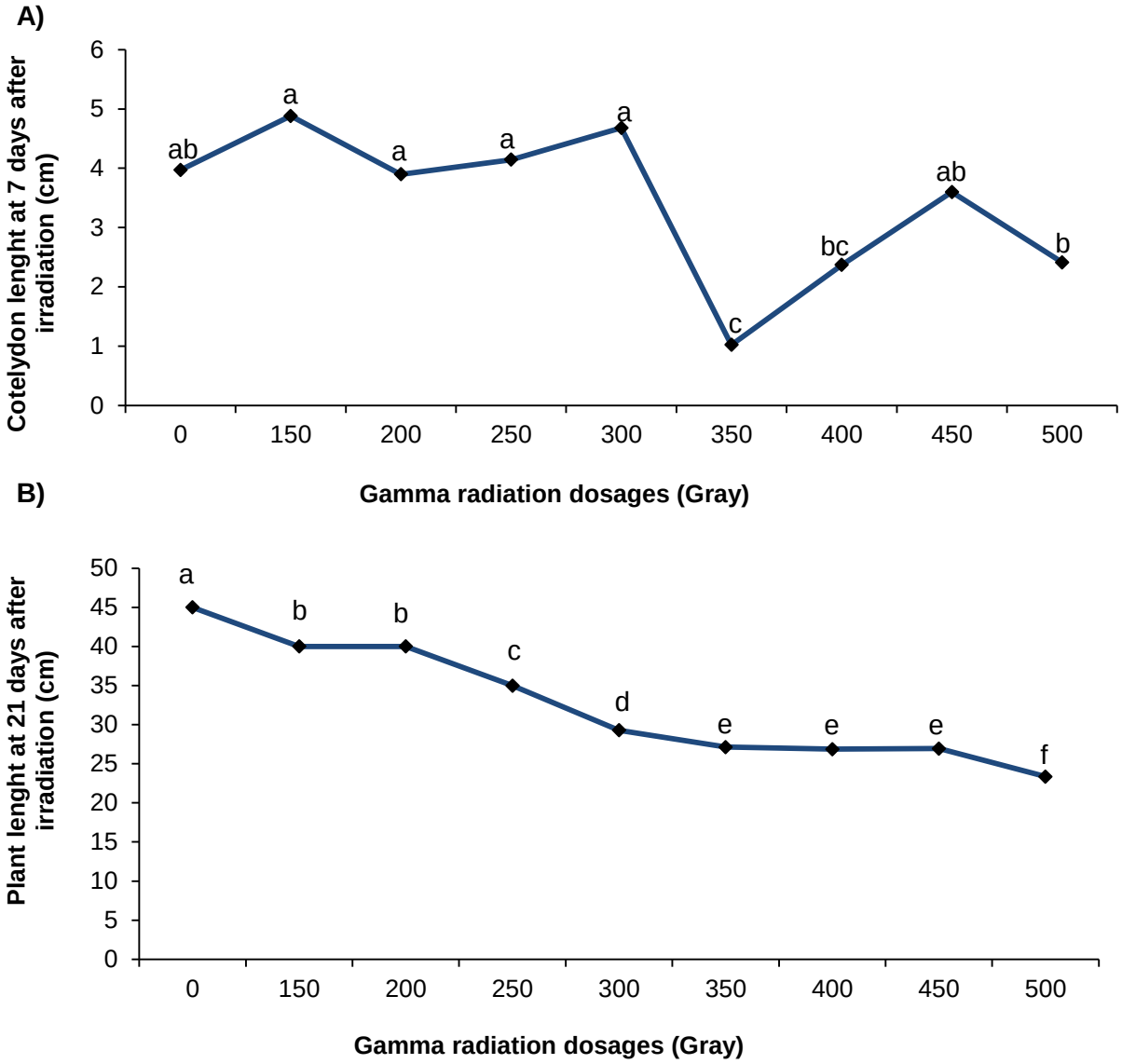


Figure 1. The effect of gamma radiation dosages in Grays (Gy) on cotyledons and plant length of maize hybrid CRN3505 at **A)** 7 days and **B)** 21 days after irradiation.

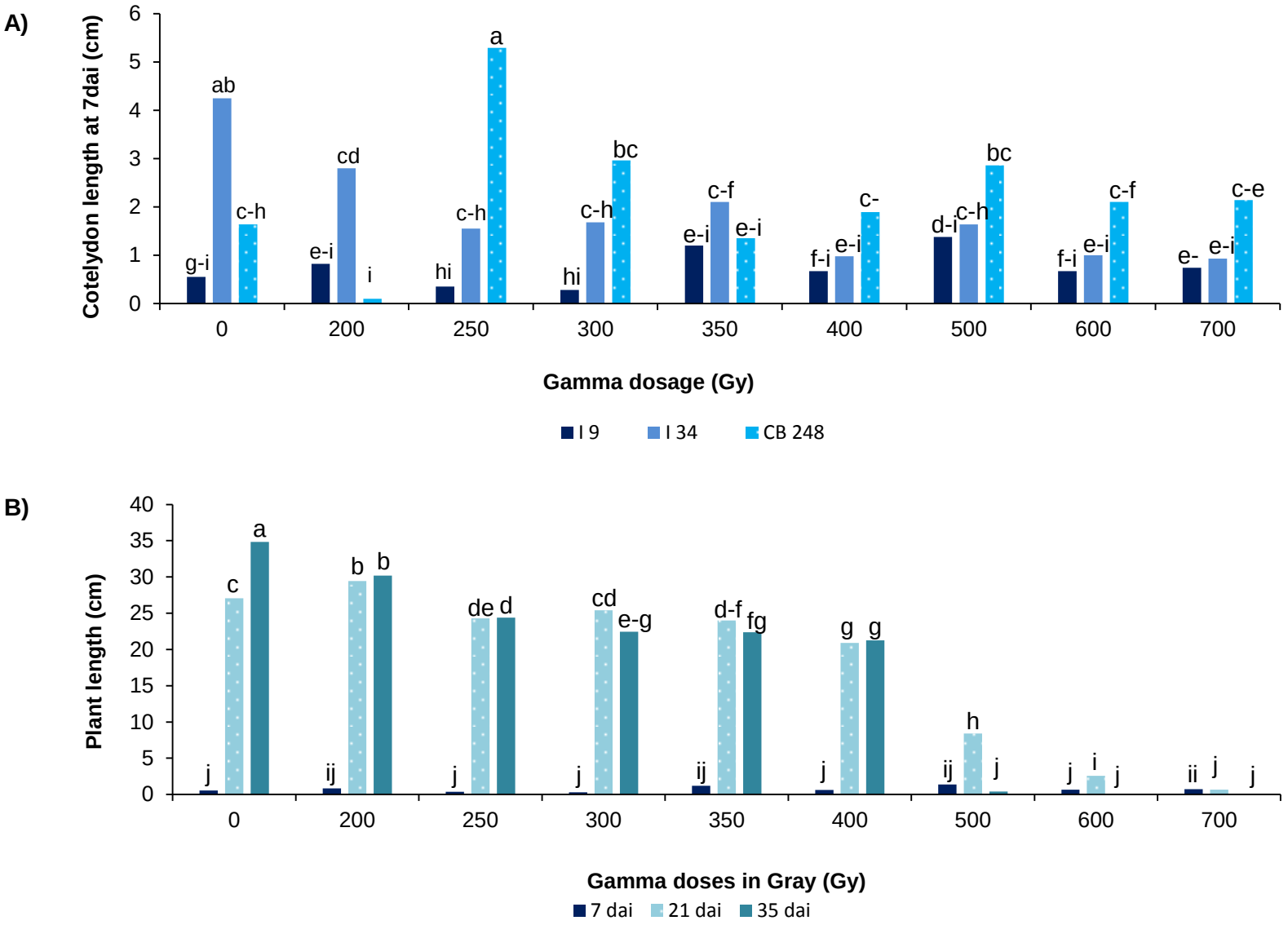
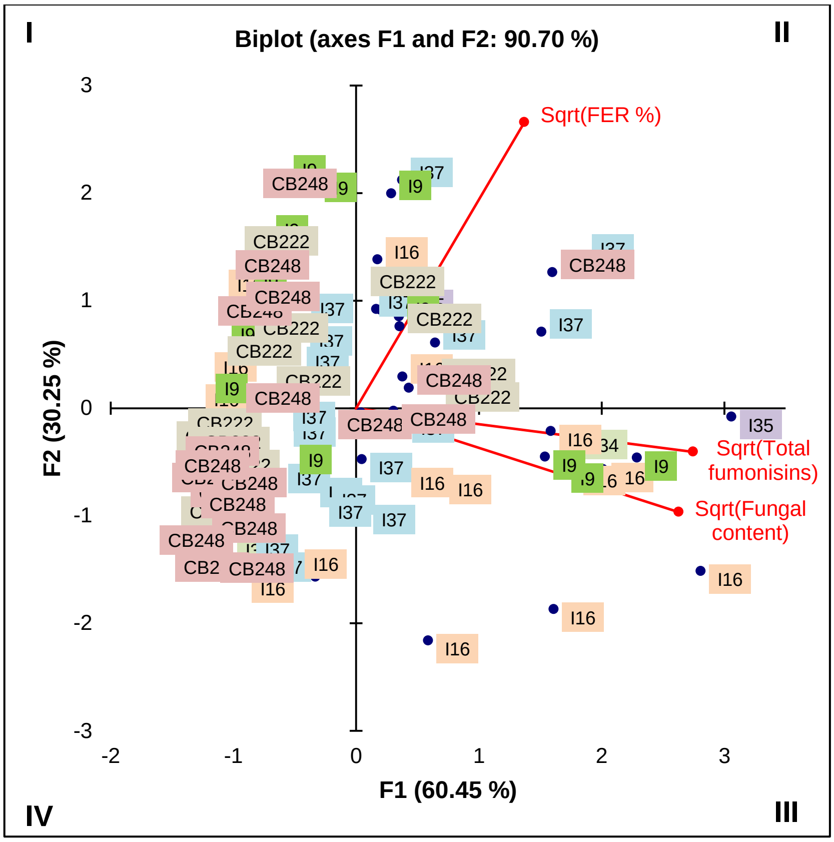


Figure 2. The effect of different gamma radiation dosages in Grays (Gy) on maize cotyledons of **A)** inbred line I-9, I-34 and CB-248 7 days after irradiation (dai) and **B)** inbred line I-9 at 7, 21 and 35 dai.

Figure 3. M_0 maize plants with symptoms of a chlorophyll phenotype (left), rough sheath phenotype (middle) and a tassel-seed phenotype (right).



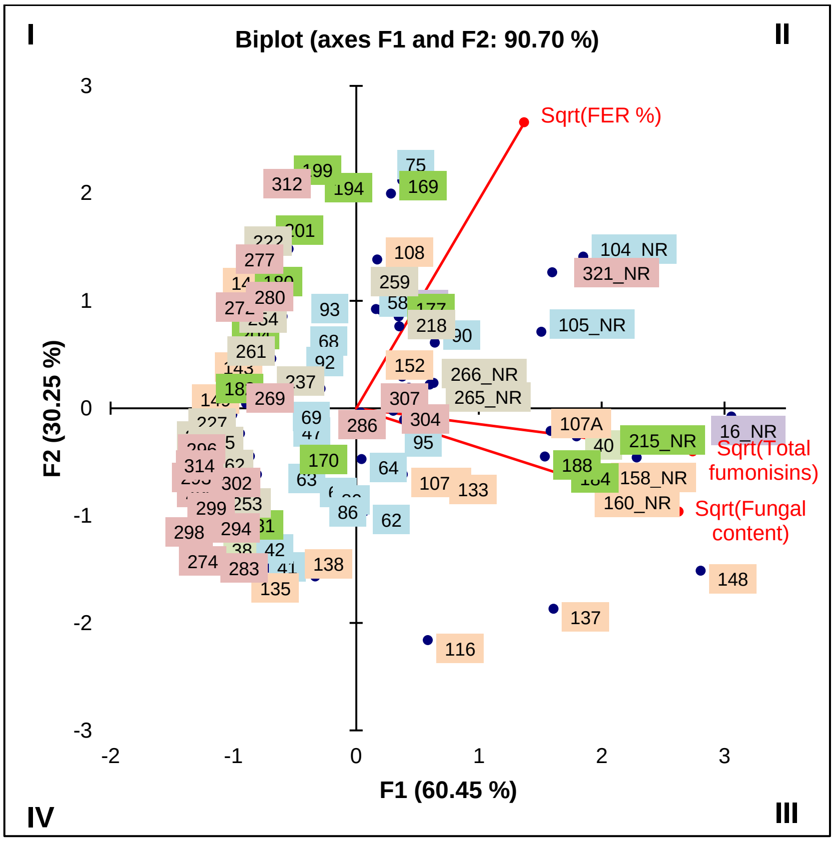


Figure 4. Principle component biplot for Fusarium ear rot, *Fusarium verticillioides* colonisation and total fumonisins in all M_4 lines (n=73) and non-irradiated inbred line controls for the 2012/13 exhibited as **A)** inbred line name and **B)** entry numbers.

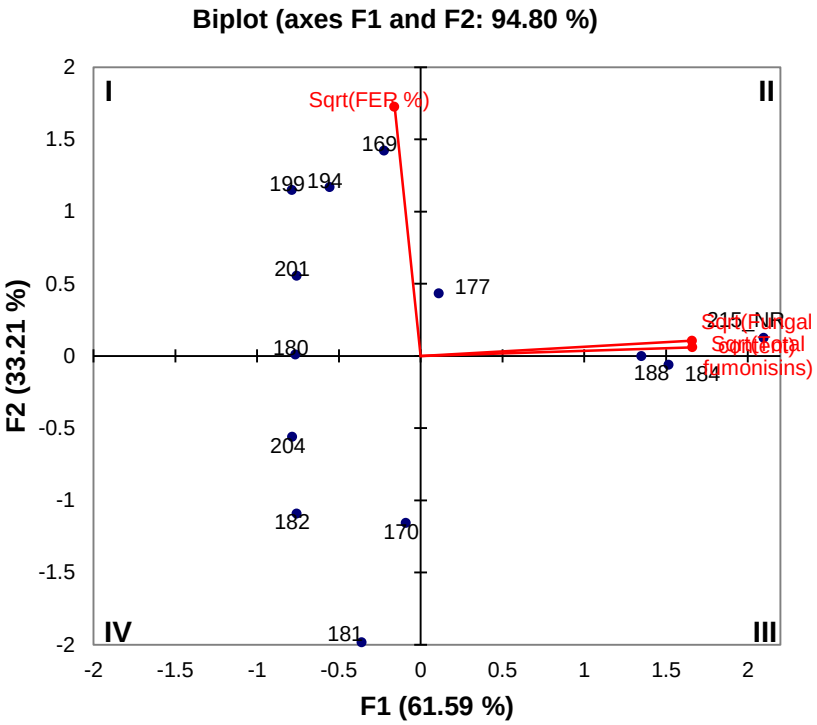


Figure 5. Principle component biplot for Fusarium ear rot, *Fusarium verticillioides* colonisation and total fumonisins of 12 M_4 inbred lines derived from the I-9 inbred line.

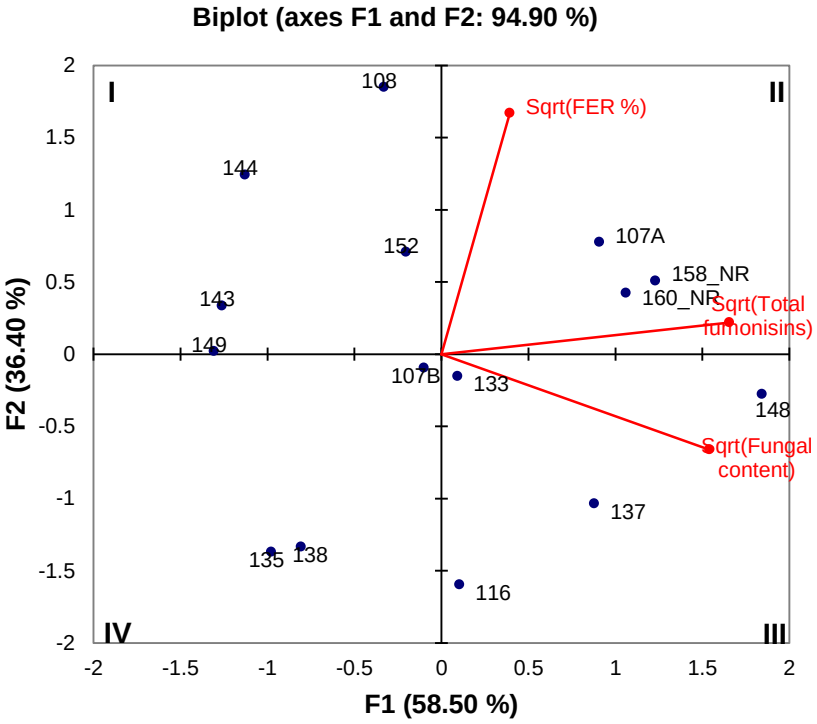


Figure 6. Principle component biplot for Fusarium ear rot, *Fusarium verticillioides* colonisation and total fumonisins of 13 M_4 inbred lines derived from the I-16 inbred line.

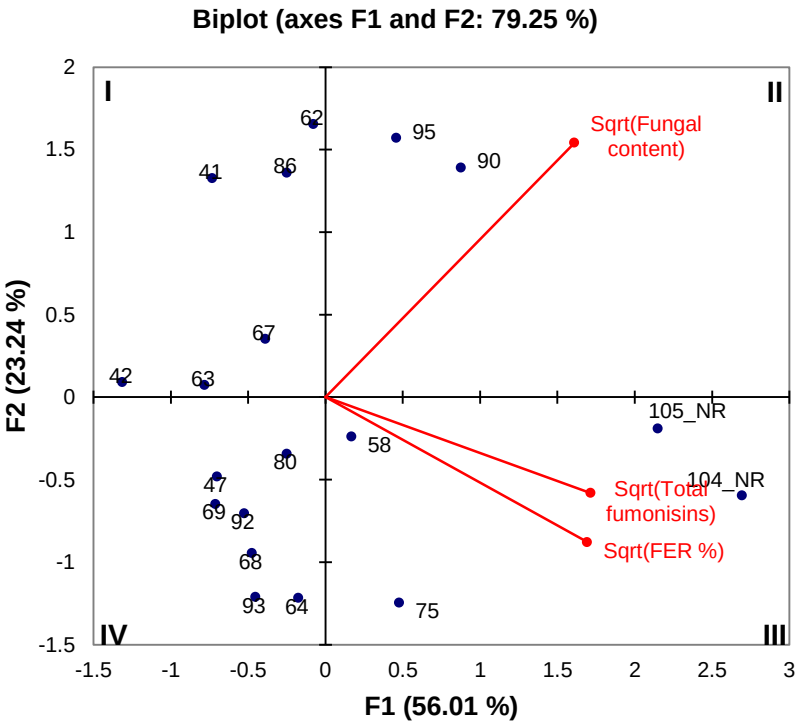


Figure 7. Principle component biplot for Fusarium ear rot, *Fusarium verticillioides* colonisation and total fumonisins of 17 M_4 inbred lines derived from the I-37 inbred line.

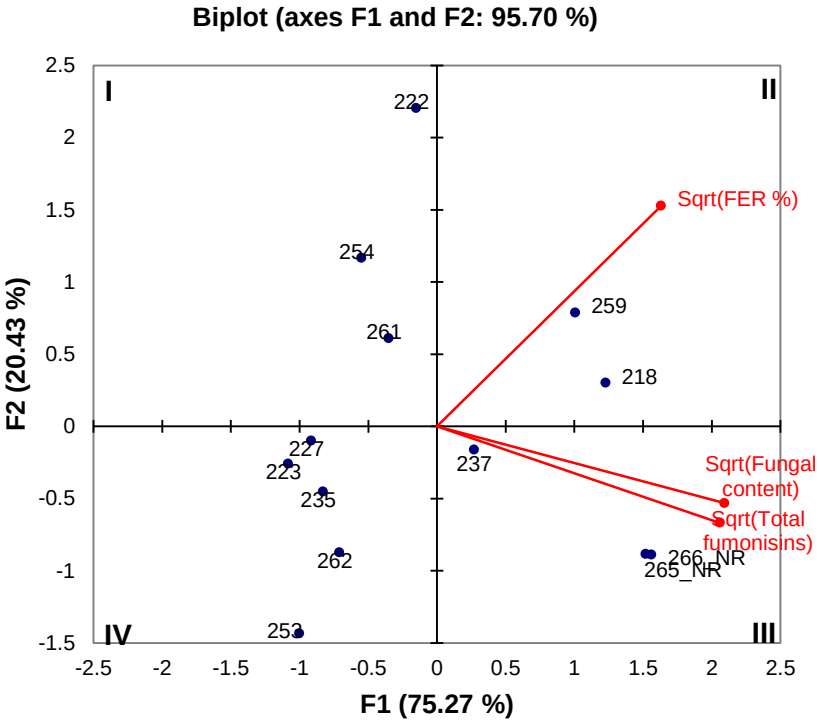


Figure 8. Principle component biplot for Fusarium ear rot, *Fusarium verticillioides* colonisation and total fumonisins of 11 M_4 inbred lines derived from the CB-222 inbred line.

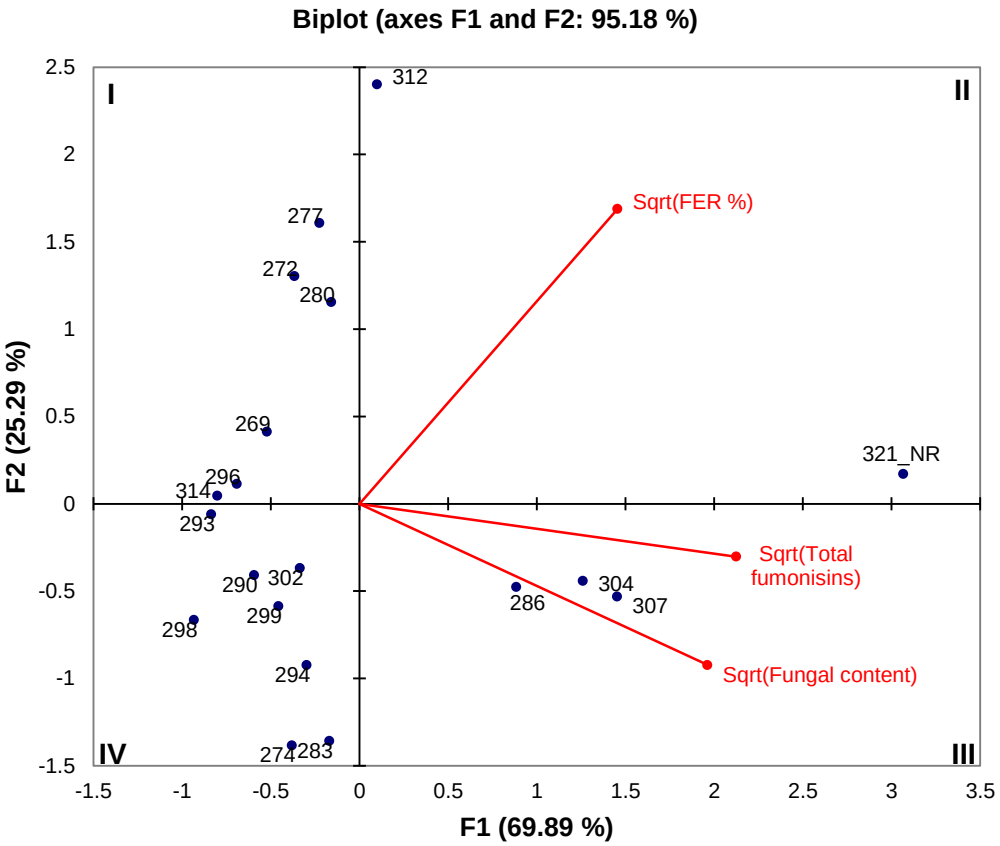


Figure 9. Principle component biplot for Fusarium ear rot, *Fusarium verticillioides* colonisation and total fumonisins of 18 M_4 inbred lines derived from the CB-248 inbred line.

CHAPTER 5

Transcriptional changes in maize following infection by *Fusarium verticillioides*

ABSTRACT

Fusarium verticillioides is a fungal pathogen of maize that causes Fusarium ear rot (FER) worldwide. The fungus reduces grain quantity and quality by destructive moulding of maize kernels, and contaminates the grain with mycotoxins. These mycotoxins, called fumonisins, are associated with harmful effects to both humans and animals. FER and fumonisins are best managed by growing maize cultivars resistant to *F. verticillioides*. Most commercial cultivars, however, are susceptible to *F. verticillioides* infection, and new resistant cultivars are yet to be developed. Conventional and marker-assisted breeding has been slow due to the low number of genes associated with resistance and the relatively small effects of quantitative resistance alleles on resistance. A proper understanding of how maize responds to infection by *F. verticillioides* may provide new insights and identify candidate genes that could be used to introduce resistance to FER and fumonisins into maize plants. In this study, transcriptional changes were investigated over 7 days in a maize inbred line inoculated with *F. verticillioides*. Defence-related transcripts associated with pathogen recognition, signalling molecules, pathogenesis-related genes, cell wall restructuring and secondary hormone-based signalling genes were induced 24 hours post inoculation (hpi). At 72 hpi, genes involved in pathogenesis, defence response to fungi, G-coupled receptor signalling and response to oxidative stress were induced. These responses, however, did not prevent *F. verticillioides* from colonising maize kernels, as the target DNA of the pathogen continued to increase. Seven days after infection, transcripts relating to G-coupled receptor signalling, oxidation-reduction, pathogenesis and cell wall organisation remained induced, while those associated with oxidative stress, defence and cell wall modification were down-regulated. The transcriptional changes suggest a slow plant response to infection by *F. verticillioides*, and imply a pathogen-associated molecular pattern response characterised by a basal plant defence mechanism.

INTRODUCTION

The hemi-biotrophic fungus *Fusarium verticillioides* (Sacc.) Nirenberg is an important phytopathogen that causes Fusarium ear rot (FER) of maize (*Zea mays* L) but also causes seedling blight and root- and stalk rot. It occurs wherever maize is produced and its presence can lead to yield losses and reduced grain quality. The fungus can exist as an endophyte in maize plants and become pathogenic under circumstances which are still poorly understood (Munkvold *et al*., 1997; Bacon *et al*., 2008; Cao *et al*., 2013). *Fusarium verticillioides* infects maize plants at different developmental stages from infected seeds, or through the roots, through the silk channel and openings created by birds, insects and mechanical damage (Warfield and Davis, 1996; Munkvold and Carlton, 1997; Munkvold and Desjardins, 1997; Yates *et al.*, 1999). Additionally, *F. verticillioides* infection of maize ears is aggravated by the production and deposition of toxic secondary metabolites, called fumonisins, in maize grain. Fumonisins have been associated with a number of harmful effects on humans (Franceshi *et al*., 1990; Rheeder *et al*., 1992; Li *et al*., 2001; Missmer *et al*., 2006) and animals (Kellerman *et al*., 1990; Gelderblom *et al*., 1994; Osuchowski *et al*., 2005).

Cultural practises such as crop rotation, reduced plants stress through proper water and nutrient management and early harvesting can limit infection of maize grain by *F. verticillioides* (Miller, 2001; Munkvold, 2003; Blandino *et al.*, 2008a, b; Abbas *et al.*, 2012). The planting of resistant cultivars, however, remains the most efficient, economically sound and environmentally friendly means to manage the fungus (Munkvold and Desjardins, 1997). The introduction of host-plant resistance into susceptible maize plants is complex, as resistance is polygenic, quantitative and strongly influenced by environmental factors (Pérez-Brito *et al*., 2001; Robertson-Hoyt *et al*., 2006; Hung and Holland, 2012; Santiago *et al*., 2015). While conventional breeding for resistance to *F. verticillioides* is a feasible strategy, the duration, cost and limited number of genes associated with resistance to *F. verticillioides* hinders the process.

Plant improvement of crops in recent years has combined conventional techniques with genomic tools and approaches (Pérez-de-Castro *et al*., 2012). The vast genomic information gained from high-throughput next generation sequencing (NGS) technologies is facilitating the direct study of the genotype and its relationship with the phenotype (Tester and Langridge, 2010). The development of high-throughput NGS methods and advances in bioinformatics have provided plant breeders with large collections of markers, high-throughput genotyping strategies, high density genetic maps and new experimental populations that can be integrated into existing plant improvement programmes (Varshney and Tuberosa, 2007a, b; Lorenz *et al*., 2011). Genomic strategies are facilitating the

detection of quantitative trait loci (QTLs), which could be employed in marker-assisted selection of improved breeding material, even in species with no previous genomic information (Varshney *et al*., 2010). Furthermore, the application of NGS in gene expression studies has provided a rich source of biological information which allows breeders to understand the molecular basis of complex plant processes and responses (Pérez-de-Castro *et al*., 2012). This has resulted in the identification of new targets for plant improvement which could be introduced or over-expressed in plants by genetic engineering.

Plants protect themselves from pathogens by means of pre-existing (constitutive) and induced structural barriers and biochemical responses (Agrios, 2005). The innate induced response is based on three important components: recognition, signalling and pathogen disablement. In return the pathogen will attempt to surpass the plant's defence responses by circumventing, blocking or overcoming them (Kearney *et al*., 1988). Pathogen recognition is mediated by plant receptors located in the apoplast, plasma membrane and the cytoplasm, such as leucine rich repeats (NBS-LLR). Initial recognition of pathogen-associated molecular patterns (PAMPs) leads to signalling pathways mediated by phosphorylation cascades that activate basal defences (Greeff *et al*., 2012), which include biochemical and metabolic changes. These responses then block off, disables or kills the pathogen by thickening the cell wall and producing antimicrobial metabolites, reactive oxygen species harmful to microbes, and enzyme inhibitors (Heath, 2000). Some of the most commonly produced metabolites include phenolic compounds, phytoalexins and flavonoids (Sekhon *et al*., 2006; Sampietro *et al*., 2013).

Gene expression, microarray and proteomic studies have investigated the mechanisms underlying the maize defence response to *F. verticillioides* infection in kernels (Kitajima and Sato, 1999; Campo *et al*,. 2004; Sekhon *et al*., 2006; Lanubile *et al*., 2010; 2012a, b; 2014). Using microarrays, Lanubile *et al*. (2010) were able to classify an exhaustive number of differentially expressed gene transcripts from a resistant and susceptible maize inbred line infected with *F. verticillioides* into 11 functional categories. These included transcripts associated with pathogen recognition and signalling, different transcription factors, phytohormones and secondary metabolites that contributes to host resistance against *F. verticillioides*. The primary means of protection against fungal infection was suggested as a constitutive defence response, as defence-related genes in a resistant line were transcribed at higher levels prior to infection, while they were induced in the susceptible line only after pathogen infection (Lanubile *et al*., 2010; 2012a,b). Similar responses to *F. verticillioides* infection were observed in both resistant and susceptible inbred lines, although the magnitude of gene induction was much greater in the resistant one (Murillo *et al*., 1997; Lanubile *et al*., 2014). Campos-Bermudez *et al*. (2013) also found no important differences in the transcriptional and metabolic profiles of resistant and susceptible

maize lines following *F. verticillioides* inoculation, and suggested that a preformed or constitutive defence mechanism may confer an advantage to the resistant line.

Understanding the response of maize plants to *F. verticillioides* infection may provide a significant insight on plant defence. Therefore, next generation RNA sequencing was used to identify the transcriptional changes associated with maize in response to *F. verticillioides* infection during the first 7 days following artificial inoculation. This study forms part of a comprehensive investigation to better understand the maize plant's response to to *F. verticillioides* and fumonisin accumulation. The current investigation explored transcriptional responses of maize from from 0-7 days after infection (dai), whereas responses between 7 and 30 dai had been reported previously by Van Zyl (2015).

MATERIALS AND METHODS

Plant material

The maize inbred line R2565y was selected for this study. The line is a commonly employed tester line originating from the Agricultural Research Council - Grain Crops Institute's previous breeding programme at Pietermaritzburg, KwaZulu-Natal. Before planting seeds were heat-sterilised in a 60˚C waterbath for 5 min (Leslie and Summerell, 2006) and germinated on damp, sterile paper towels incubated at 27ºC for 5 days. Germinated seeds, free of fungal contamination, were then planted directly into 15-L plant bags filled with organic coconut coir growth medium (Vegtech 2000, Cape Town, South Africa) in a greenhouse at Welgevallen experimental farm at Stellenbosch University, Stellenbosch, South Africa. Seedlings were irrigated daily with approximately 500 ml non-fertilised water for 1 week, then irrigated twice daily with a nutrient solution consisting of 0.354 mg L^{-1} KNO_3, 0.068 mg L^{-1} KH_2PO_4, 0.115 mg L^{-1} $NH_4H_2PO_4$, 0.4 mg L^{-1} $Ca(NO_3)2.2H_2Oz$, 0.009 mg L^{-1} Libfer (Fe-EDTA), 0.002 mg L^{-1} manganese sulphate, 0.0012 mg L^{-1} zinc sulphate, 0.0012 mg L^{-1} solubor, 0.0002 mg L^{-1} copper sulphate, and 0.0001 mg L^{-1} sodium molibdate at an electrical conductivity (EC) of 1.5 and at a pH of 6.5. After 5 weeks the EC of the nutrient solution was adjusted to 2.5 and the pH maintained at 6.5. Leaf samples were collected from each plant at the 4-leaf stage for DNA fingerprinting at CenGen PTY, Western Cape, South Africa to ensure the genetic homology of individual plants. Emerging ear shoots were covered with a transparent shoot bag before flowering, and pollinations performed at 50% silk emergence.

Inoculation of maize ears

The *F. verticillioides* culture MRC 826, isolated from infected maize in the former Transkei region (Eastern Cape Province), was used for artificial inoculation of the maize inbred line

R2565y. Inoculum was prepared by growing the culture on potato dextrose agar (PDA) for 10 days at 25^{o}C, then the mycelia were transferred into Armstrong liquid medium (Booth, 1971). After 3-4 days incubation at 25ºC in an incubator shaker at 100 revolutions min^{-1} (rpm), the conidia were separated from the mycelia by filtration though two layers of sterile cheesecloth. The conidial suspension was then centrifuged for 10 minutes at 3 500 G, and the conidial pellet washed twice with 45 ml deionized, autoclaved H_2O. After the second washing step, the conidial concentration was adjusted to 1 x 10^{6} conidia ml^{-1} using a haemocytometer. The inoculum was kept at 4^{o}C prior to and during the inoculation process and inoculum.

The primary ear of each maize plant was inoculated with *F. verticillioides* MRC 826 at 35 days after pollination. Two ml of the conidial suspension was injected down the silk channel of ears using a sterile needle and syringe, while control plants were inoculated with 2 ml of sterile, distilled water. Three maize ears were inoculated for each time point that ears would be collected (0, 24, 48, 72 hrs and 7 days). The collected ears were flash frozen in liquid nitrogen at the time of sampling and stored at -80ºC until the grain was processed. Following storage, the maize kernels were milled to fine flour in liquid nitrogen using 0.1% DEPC-treated mortars and pestles. Milled maize samples were stored at -80ºC until biological analyses were performed.

FER severity, *Fusarium verticillioides* and fumonisin quantification

The fungal contamination of maize grain at each sampling time point was determined by absolute quantification of *F. verticillioides* target DNA, as described by Boutigny *et al*. (2012). Visual assessment of disease severity as well as fumonisin content of the grain was not performed as grain was only sampled up to 7 dai.

RNA extraction and quality control

Total RNA was extracted from maize kernels using a crude, sodium dodecyl sulphate (SDS)/TRIzol (Invitrogen, Carlsbad, USA) RNA isolation method (Wang *et al.*, 2011), with modifications. Maize flour (0.2 g) was thoroughly mixed in 400 uL extraction buffer (100 mM Tris-HCL (pH 9), 2% β-mercaptoethanol w/v) by vortexing, and then incubated at room temperature for 15 min. After incubation 20 µL 20% (w/v) SDS was added, the samples inverted a few times, and the suspension incubated for 5 min more at room temperature. The samples were centrifuged at 13.3 G for 10 min at 4ºC, then the aqueous phase was transferred to new micro-centrifuge tubes. Two volumes of TRIzol were added to the aqueous phase, thoroughly mixed by vortexing, and incubated at room temperature for 10 min.

Following incubation, 240 µL chloroform was added to the samples, mixed well and centrifuged at 13.3 G for 10 min at 4ºC. The aqueous phase (700 µL) of each sample was once more transferred to a new micro-centrifuge tube, and an equal volume of isopropanol was added. Samples were mixed by inversion and precipitated at -20ºC for 20 min, then they were centrifuged at 13.3 G for 10 min at 4ºC. The supernatant was then discarded and the remaining pellet re-suspended in 400 µL DEPC-treated H_2O, followed by the addition of 400 µL citrate buffer-saturated phenol (pH4.3):chloroform (1:1). The samples were once more thoroughly mixed and centrifuged at 13.3 G for 10 min (4ºC). The aqueous phase of each sample was then transferred to a new micro-centrifuge tube and combined with 40 µL 3 M sodium acetate (pH 4.8) and 800 µL ice-cool ethanol, mixed by inversion, and precipitated at -80ºC for 30 min. After precipitation the samples were centrifuged at 13.3 G for 10 min (4ºC) and the supernatants transferred to new tubes. Pre-cooled 70% ethanol (500 uL) was added to each sample, centrifuged and the supernatant discarded. The pellets were air-dried at room temperature for 10 min then dissolved in 50 µL DEPC-treated H_2O and stored at -80ºC.

The quantity of total RNA obtained from maize kernels was determined with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). The sample requirements for RNA-Sequencing (RNA-Seq) and quantification included ≥3 µg total RNA at a concentration of ≥50 ng μL^{-1} and a sample purity of $OD_{260/280}$=1.8~2.2, RNA 25S:18S ≥1 and a RIN value of ≥6.5. Equal quantities of the three biological replicates representing each treatment and time point were pooled to obtain a final quantity of 4.5 µg total RNA. The quality and quantity of RNA samples was confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA) at the Central Analytical Facilities (CAF), Stellenbosch University.

Library preparations and sequencing of the transcriptomes

Total RNA samples were sent to Novogene Bioinformatics Technology CO. Ltd. (Beijing, China) for RNA-Seq. Quality measurements of the RNA included the visualisation of total RNA on a 1% agarose gel to determine degradation and contamination. Furthermore, the purity was determined using a NanoPhotometer® spectrophotometer (IMPLEN, Westlake Village, CA, USA) while the Qubit® 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA) accurately determined RNA concentration. Lastly, RNA integrity was determined by the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Total RNA samples (3 µg) were used to isolate poly(A) mRNA using Oligo(dT) magnetic beads for the preparation of a RNAseq library using the TruSeq RNA Sample Prep Kit v2 (Illumina Inc., San Diego, CA, USA). Libraries were sequenced on an Illumina Hiseq 2000/2500 platform (Illumina Inc., San Diego, CA, USA) and 100 bp/50 bp single-end reads were generated.

Data analysis

Standard bioinformatic analysis was performed by Novogene Bioinformatics Technology CO. The raw image data was first transformed to sequence data by base calling. During this process, raw reads were filtered by discarding reads that contained adapters, reads with more than 10% “N”-nucleotide designation and those of low quality. Concurrently, Phred quality scores (Q), logarithmically related to the base calling error, were calculated. The percentage Q20 related to one incorrect base pair (bp) in 100 bp (99.0% correct base calling rate), while the percentage Q30 related to one incorrect base pair in 1 000 bp (99.9% correct base calling rate). The guanine-cytosine (GC) content of clean reads were also determined. The GC distribution was evaluated to detect potential AT/GC separation which affects gene expression quantification. Only clean reads of a high quality were subjected to bioinformatic analyses.

Appropriate software was selected according to the characteristics of the reference genome, and the maize inbred line B73 was used as reference genome. The programmes Bowtie v2.0.6 (Johns Hopkins University, Centre for Computational Biology (CCB); http://bowtie-bio.sourceforge.net/index.shtml) and TopHat v2.0.9 (Johns Hopkins University, CCB; http://ccb.jhu.edu/software/tophat) were utilised to align the sequence reads to the reference sequence, which was directly obtained from the genome website (http://www.maizegdb.org/assembly). The algorithm employed by Tophat2 involved the alignment of reads to a reference genome, the mapping of reads to exons, as well as the segmentation of reads and subsequent mapping to the adjacent exons (splice junctions). For unigene digital gene expression (DGE), Bowtie v0.12.9 was used to aligned single-end clean reads to the unigene sequences. The visualisation of the mapping status of the reads was done with Integrative Genomics Viewer (IGV) (BROAD Institute - https://www.broadinstitute.org/igv/).

Gene expression levels were quantified by HTSeq v0.5.as the number of reads that mapped to genes or exons when compared to the maize B73 reference genome. The read count indicated the gene’s expression level, as well as its length and sequencing depth. The gene expression level was standardised between samples by the Reads Per Kilo Bases Per Million Reads (RPKM) method (Mortazavi *et al.*, 2008), which eliminates the influence of different gene lengths and sequence discrepancies. Therefore, the calculated gene expression could be directly used for comparing the differences in gene expression among samples. In general, 0.1 or 1 RPKM was set as the threshold to judge whether the gene was being expressed or not. Differentially expressed transcripts between inoculated and control kernels were determined by using the DEGSeq 1.12.0 R package. The p-values were corrected (q-values) (Benjamini and Hochberg, 1995), and the threshold for significant

differential expression was determined at q value <0.005 and $|\log_2$ (fold-change) | ≥1, as the biological replications were pooled. The differentially expressed genes (DEGs) were annotated with Blastp (protein-protein BLAST). Gene Ontology (GO) enrichment analysis was performed using the Singular Enrichment Analysis (SEA) tool of AgriGO (http://www.bioinfo.cau.edu.cn/agriGO/analysis.php) with the maize genome V5a transcript ID as background by applying a Fisher statistical test with the Yekutieli multi-test adjustment. GO terms with corrected p value <0.05 were considered significantly enriched by differential expressed genes.

RESULTS

RNA-sequencing of maturing kernels challenged with *Fusarium verticillioides*

Raw and clean reads were produced by RNA-Seq following inoculation of maize kernels with *F. verticillioides* (Table 1). The percentage Phred quality scores (Q20 and Q30) were <98.8 and 96.2%, respectively. The GC content of inoculated and control samples ranged from 52.1-56.2% (Table 1). The clean reads were successfully mapped to the reference genome of *Z. mays*, with the total mapped reads/fragments (TMR) being more than 87% (Table 2). The multiple mapped reads/fragments (MMR) of the samples were between 9 and 17%, although this should be no more than 10% (Table 2). In total, 6 827 679 putative novel expressed loci were found at Day 0. For the control and inoculated plants, respectively, a total of 5 846 093 and 6 419 653 genes were expressed at 24 hrs post inoculation (hpi), 7 646 444 and 7 151 941 at 48 hpi, 8 102 010 and 6 798 996 at 72 hpi, 7 606 186 and 6 313 640 at Day 7 (Table 2). The mapped regions had been classified as exons, introns and intergenic regions. The exon-mapped reads were more than the introns and intergenic regions for all of the sequenced samples. The differentially expressed genes detected at Day 0 were 6 477, while 6 078 and 6 543 were detected at 24 hpi, 6 448 and 6 275 at 48 hpi, 6 490 and 6 142 at 72 hpi, and 6 352 and 6 556 at Day 7, 6 521 of the control and inoculated plants, respectively.

In total, 706 known protein-coding genes were differentially expressed in the inoculated kernels 24 hpi, with 384 genes being up-regulated and 322 genes being down-regulated (Fig. 1A). At 48 hpi 188 protein-coding genes were differentially expressed, with 59 genes up-regulated and 129 genes repressed (Fig. 1B). Seventy-two hours after inoculation, 379 protein-coding genes were differentially expressed with 158 being up-regulated and 221 being down-regulated (Fig. 1C). A total of 172 known protein-coding genes were differentially expressed in the inoculated kernels at Day 7, with 71 being up-regulated and 101 being repressed (Fig. 1D). Venn diagrams indicated that 105 differentially expressed genes (DEG's) overlapped between 24 and 48 hpi, 100 were common between 48 and 72

hpi, while 63 DEG's were common between 72 hpi and Day 7 (Fig. 2A-C). The annotated DEGs with a GO classification (Supek *et al.,* 2011) were sorted manually into four classical defence categories, i.e. the activation of signalling networks, differential expression of defence-related genes, differential expression of hormone signalling-related genes, and the differential expression of secondary metabolism-related genes (Figs. 3-6).

Expression of plant genes in response to *Fusarium verticillioides*

Pathogen recognition: Receptor-coding genes were induced by *F. verticillioides* in maize kernels 24 hpi (Table 3). Three probable leucine-rich repeat (LRR) receptor-like serine/threonine protein kinases (PKs) (GRMZM2G011526; GRMZM2G126858; GRMZM2G176922) were strongly (2.93; 3.58; 2.19 $\log_2$-fold) up-regulated. A putative receptor-like protein kinase family protein (GRMZM2G006080) and brassinosteroid signalling positive regulator-related protein (GRMZM5G812774) (Lanubile *et al*., 2014) were, however, down-regulated (1.13; 1.95 $\log_2$-fold), respectively, at 24 hpi. No receptor-coding genes were induced 48 hpi (Fig. 4), while the probable LRR receptor-like serine/threonine protein kinase (GRMZM2G176922) was repressed 1.95 $\log_2$-fold at 72 hpi. No receptor-coding genes were induced by *F. verticillioides* in maize kernels at Day 7 (Table 3).

The activation of signalling networks: Several signalling networks, including protein kinase-, calcium-, guanosine triphosphate (GTP)- and redox-signalling were induced following *F. verticillioides* inoculation (Table 4, 5 and 6). At 24 hpi a mitogen-activated protein kinase kinase kinase (MAPKKK) (GRMZM2G093316), a MAPK homolog MMK1 (GRMZM2G147811), a MAPK homolog NTF4 (GRMZM2G151236; 2.12 $\log_2$-fold) and a M2K1 MAPKK (GRMZM2G312970) (Lanubile *et. al*., 2014) were up-regulated (Table 4). Furthermore, an uncharacterised protein kinase (GRMZM2G094039; 2.71 $\log_2$-fold), two YAK1 protein kinase family proteins (GRMZM2G092959; GRMZM2G311051) (Lanubile *et al*., 2014), a phosphatidylinositol 3- and 4-kinase family protein (GRMZM2G322846), a phosphatidylinositol-3-kinase (GRMZM2G103721) and a 1-phosphatidylinositol 4-kinase (GRMZM2G114162), were also up-regulated at 24 hpi. Additionally, a serine/threonine-protein kinase (AC218998.2_FG007) was strongly up-regulated (1.98 $\log_2$-fold) and a casein kinase I isoform (GRMZM2G075683) was also up-regulated. Conversely, a MAPK activating protein (GRMZM2G025242) and a SNF1-related protein kinase regulatory subunit protein (GRMZM5G897067) were down-regulated (Table 4).

At 48 hpi, the MAPKKK (GRMZM2G093316) and two putative DUF869 domain containing family proteins (GRMZM2G142019; AC205419.3_FG001) were repressed (Table 4). At 72 hpi the expression of all protein kinase signalling proteins were reduced in comparison to the control, with the exception of a SNF1-related protein kinase regulatory

subunit (GRMZM5G897067). A putative MAPKKK family protein kinase isoform 2 (GRMZM2G093316), a MAPK homolog NTF4 (GRMZM2G151236; -1.9 log_2-fold) and a MAPK homolog MMK1 (GRMZM2G147811; -1.9 log_2-fold) were all down-regulated (Fig. 3). Similarly, the expression of a putative DUF869 domain-containing family protein (GRMZM2G142019) was strongly reduced (-2.05 log_2-fold), while another putative DUF869 domain containing family protein (AC205419.3_FG001) was repressed, along with a phosphatidylinositol 3-kinase gene (GRMZM2G103721) and a phosphatidylinositol 4-kinase alpha gene (GRMZM2G114162). Downstream signalling networks continued to be regulated at Day 7, but a MAPKKK (GRMZM2G093316), an uncharacterised protein kinase (AC218998.2_FG007), calcium-dependent protein kinase isoform 11 (GRMZM2G032852) and a putative 1-phosphatidylinositol-4-phosphate 5-kinase / zinc ion binding family (GRMZM2G153722) were up-regulated in the infected kernels (Table 4).

Calcium homeostasis regulators were induced at all the time points (Table 5). Twenty four hours after inoculation a calmodulin binding protein (GRMZM2G167982) was strongly induced (2.91 log_2-fold), along with calmodulin binding protein GRMZM2G052875, while a third calmodulin binding protein (GRMZM2G010868) was down-regulated. A gene encoding calmodulin 5 (CAM5) (GRMZM2G152891), a putative uncharacterised protein involved in cellular calcium ion homeostasis (GRMZM2G114775) and a chaperonin 20 protein (GRMZM2G091189) were all repressed, while two genes encoding calreticulin 1 (CRT1) (GRMZM2G074687; GRMZM2G358059) and calreticulin 3 (CRT3) (GRMZM2G305115) were both up-regulated (Fig. 3). At 48 hpi CRT1 (GRMZM2G358059), CRT3 (GRMZM2G305115) and an EF hand protein (GRMZM2G145905), which forms part of the calcium signalling pathway, were all down-regulated (Fig. 4), while a calmodulin binding protein (GRMZM2G052875) and a gene encoding calreticulin 3 (GRMZM2G305115) were also down-regulated 72 hpi (Fig. 5). A calcineurin-like phosphoesterase family protein (GRMZM2G163494) was, however, strongly induced (2.7 log_2-fold) at 72 hpi. Seven days after inoculation all genes involved in calcium homeostasis were up-regulated. These included a calcium-transporting ATPase gene (GRMZM2G391042), as well as CRT1 (GRMZM2G358059) (Table 5).

Guanosine triphosphate (GTP) signalling-related genes were induced as early as 24 hpi and remained induced up to 7 days in the inoculated kernels (Table 6). A gene encoding for GTP binding (GRMZM2G365862) was strongly induced (4.56 log_2-fold) 24 hpi, while two other GTP binding genes were repressed (GRMZM2G071790; GRMZM2G172932). An uncharacterised protein with a role in G-protein signalling (GRMZM2G159744) was down-regulated 1.89 log_2-fold 48 hpi, while a G-protein-coupled receptor and uncharacterised proteins involved in G-protein binding (GRMZM2G427424; GRMZM2G028313;

GRMZM2G157462) were also down-regulated 72 hpi. One GTP signalling-related gene (GRMZM2G151195) was up-regulated (1.02 log_2-fold change) at Day 7 (Table 6).

The expression of genes involved in redox signalling was generally reduced in inoculated kernels 24 hpi (Table 6). The expression of two glutathione S-transferase (GST) genes (GRMZM2G044383; GRMZM2G129357) were reduced 24 hpi, with a GST gene (GRMZM2G044383) being up-regulated (1.13 log_2-fold) 48 hpi. This gene remained induced 1.11 log_2-fold at 72 hpi, along with a probable glutathione S-transferase GSTU1 gene (GRMZM2G335618), which was also induced. A respiratory burst oxidase protein F (GRMZM2G169201) was induced at 24 hpi and a putative peroxiredoxin (GRMZM2G129761) and peroxiredoxin-5 (GRMZM2G036921) was down-regulated (Fig. 3). Moreover, two uncharacterised proteins (GRMZM2G154828; GRMZM2G154870, 2.25 log_2-fold), designated as cytochrome P450 superfamily protein (Lanubile *et al*., 2014), were induced 24 hai, while four putative cytochrome P450 superfamily proteins (GRMZM2G067225; GRMZM2G114988; GRMZM2G135536; GRMZM2G139874) were all repressed. A probable glutathione S-transferase GSTU6A gene (GRMZM2G028821) was also strongly down-regulated (2.32 log_2-fold) 48 hpi (Fig. 4), while a putative cytochrome P450 superfamily protein (GRMZM2G114988) was induced. A respiratory burst oxidase protein E (GRMZM2G147966) was repressed 72 hpi while a probable peroxiredoxin (GRMZM2G129761) and peroxiredoxin-5 (GRMZM2G036921) were both induced (Fig. 5). Similarly, two putative cytochrome P450 superfamily proteins (GRMZM2G057086; GRMZM2G114988) were also induced. A single gene encoding for thioredoxin (GRMZM2G144653) was repressed (1.05 log_2-fold) at Day 7 (Table 6).

The expression of defence-related genes: WRKY transcription factors, which are responsible for the activation of downstream defence genes, were not differentially expressed in maize kernels inoculated with *F. verticillioides* at any of the infection time points evaluated. However, two MYB transcription factor 3B genes (GRMZM2G078820; GRMZM2G145444) and a transcription factor MYC7E gene (GRMZM2G001930) were down-regulated at 24 hpi (Table 7). No nucleotide binding site (NBS)-LRR proteins, encoding for resistance genes, were expressed at 24 hpi, but a RPM1-interacting protein 4 (GRMZM2G099745) was up-regulated 48 hpi. No other NBS-LRR proteins were expressed at any other time point. A leucine-rich repeat resistance protein (GRMZM2G107872), a polygalacturonase inhibiting protein 2 (PGIP2) (GRMZM2G099295) and a disease resistance response protein 206 (GRMZM2G059706) were all repressed at 24 hpi. A zeamatin-like protein (GRMZM2G374971), documented as an antifungal protein of maize, was induced at 72 hpi (Table 7).

At 24 hpi, three pathogenesis-related (PR) proteins (GRMZM2G092474; GRMZM2G465226; GRMZM2G039639) were strongly induced (1.8; 2.19 and 2.66 log_2-fold), while one PR protein transcriptional activator PTI6 (GRMZM2G129777) was down-regulated when compared to the water-inoculated control (Table 7). β-1,3-glucanases (PR2); including a glucan endo-1,3-beta-glucosidase precursor (GRMZM2G046459), an endo-beta-1,3-1,4 glucanase II (GRMZM2G137535) and a glucan endo-1,3-beta-glucosidase-like protein 3 (GRMZM2G143791) were also down-regulated (Fig. 10). One chitinase protein (PR3), an endo-1,4-beta-xylanase (AC204711.3_FG002), was repressed in inoculated kernels 24 hpi. Three serine protease inhibitors (PR6) were differentially expressed. A Bowman-Birk type trypsin inhibitor (GRMZM2G075315) was up-regulated, while a Bowman-Birk type trypsin inhibitor-like (GRMZM2G114552) and a cysteine proteinase 1 gene (GRMZM2G098298) were down-regulated. A win1 isoform gene (GRMZM2G117989), designated as PR4 by Lanubile *et al*. (2014), was induced 24 hpi. The expression of two peroxidases (PR9) (GRMZM2G361475; GRMZM2G108153) were down-regulated, another peroxidase family protein (GRMZM2G110289) strongly induced (2.95 log_2-fold) and two defensin-like proteins (PR12) (GRMZM2G046532; GRMZM2G368861) up- and down-regulated, respectively. Two lipid-transfer proteins (PR14) (GRMZM2G083725; GRMZM2G164440), along with several non-specific lipid transfer proteins (GRMZM2G039383, 1.96 log_2-fold; GRMZM2G081464; GRMZM2G101958; GRMZM2G126397; GRMZM2G137329) were all repressed 24 hpi (Table 7).

At 48 hpi, an endochitinase (GRMZM2G453805) and a xyalanse inhibitor protein (GRMZM2G053206) were down-regulated in inoculated kernels (Table 7). Similarly three PR5 genes; a thaumatin domain family protein (PR5) (GRMZM2G092474), a thaumatin-like gene (GRMZM2G402631, -4.54 log_2-fold) and a thaumatin-like protein 21 (GRMZM2G039639; -2.44 log_2-fold); were also down-regulated (Fig. 4). Serine proteinase inhibitor (PR6)-encoding genes, Bowman-Birk-type trypsin inhibitor (GRMZM2G075315; -2.04 log_2-fold), a cysteine proteinase inhibitor (GRMZM2G012160) and a putative cystatin (GRMZM2G401328) were all down-regulated in inoculated kernels compared to the non-inoculated kernels. A lipid transfer protein (GRMZM2G083725) and two non-specific lipid transfer proteins (GRMZM2G081464; GRMZM2G137329) were, however, induced at this time point.

Two PR2 genes, an endo-beta-1,3-1,4 glucanase II (GRMZM2G137535) and a glucan endo-1,3-beta-glucosidase (GRMZM2G125032; 2.09 log_2-fold change), were induced 72 hpi, along with two PR3 genes encoding a xylanase inhibitor protein 1 (GRMZM2G447795) and an endochitinase A (GRMZM2G051943; 2.27 log_2-fold) (Fig. 5). Furthermore, a thaumatin domain family protein (PR5) GRMZM2G092474), an uncharacterised protein involved in pathogenesis (GRMZM2G041039) and a defensin-like 2

protein (GRMZM2G368861) were also up-regulated (Table 7). Serine protease inhibitors (PR6) genes encoding a Bowman-Birk type trypsin inhibitor-like (GRMZM2G114552), a Bowman-Birk type wound-induced proteinase inhibitor WIP1 (GRMZM2G011523) and a cystatin (GRMZM2G148925) were all induced at 72 hpi. In addition, a peroxidases (PR9) peroxidase 5-like (GRMZM2G177792), a peroxidase SPC4 precursor (GRMZM2G361475) (Table 7), two lipid transfer proteins (PR9) (GRMZM2G083725; GRMZM2G164440) as well as four non-specific lipid transfer proteins (GRMZM2G010868; GRMZM2G081464; GRMZM2G101958; GRMZM2G137329) were also induced (Fig. 5).

At 7 dai very few defence related genes were differentially expressed and most were down-regulated. The repressed transcripts included two serine proteases; encoding a Bowman-Birk type trypsin inhibitor (GRMZM2G114552) and a putative cystatin (GRMZM2G401328); a cytosolic ascorbate peroxidase (GRMZM2G137839), a putative lipid transfer protein (GRMZM2G164440) and three non-specific lipid transfer proteins (GRMZM2G039383; GRMZM2G081464; GRMZM2G137329) (Table 7).

Other defence-related genes differentially expressed in maize kernels following inoculation with *F. verticillioides* were ABC transporter encoding genes, a glutamate decarboxylase, genes associated with cell wall strengthening and polyphenol oxidases (PPOs) (Table 8). Twenty four hai, an ABC1 family protein (GRMZM2G140917) was up-regulated while several genes associated with cell wall strengthening, including a 1,3-beta-glucan synthase-like (GSL) 8 (GRMZM2G326643), GSL10 (GRMZM2G453794), a gene encoding GSL12 (GRMZM2G430680) and a callose synthase gene (GRMZM5G840560), were also induced. An ABC transporter A family member (GRMZM2G355523, 2.23 $\log_2$-fold) a GSL11 (GRMZM2G084802, 5.2 $\log_2$-fold) and a GSL12 encoding gene GRMZM2G180951, 2.36 $\log_2$-fold) were strongly induced 24 hpi. Additionally, a 1,4-alpha-glucan branching enzyme (GRMZM2G073054) and a starch branching enzyme 3 gene (GRMZM2G032628) were induced, while expansin precursor A2 (EXPA2) (GRMZM2G339122, -2.05 $\log_2$-fold), EXPA7 (GRMZM2G445169), EXPB3 (GRMZM2G176595) and EXPB14 (GRMZM2G148485) were all repressed 24 hpi.

A putative polyphenol oxidase family protein (AC209206.3_FG014), a defence-associated gene (Li and Steffens, 2002), was down-regulated while a glycine-rich cell wall structural protein 2 (GRMZM2G067315) was induced at 24 hpi but down-regulated 48 hpi. A 1,3-beta-glucan synthase-like 8 (GRMZM2G326643) was down-regulated 48 hpi and remained down-regulated at 72 hpi. The EXPA7 (GRMZM2G445169) was induced at 48 hpi, while two EXPB3 genes (GRMZM2G094990, 1.94 $\log_2$-fold; GRMZM2G176595), an EXPB14 gene (GRMZM2G026956) and a beta-expansin 2 precursor gene (GRMZM2G148485) were all induced at 72 hpi. Conversely, an alpha-glucan branching enzyme 3B gene (GRMZM2G088753) and the starch branching enzyme 3 gene

(GRMZM2G032628) were down-regulated. At Day 7, a gene encoding for an ABC1 family protein (GRMZM2G140917) was up-regulated while precursors of expansin, EXPA2 (GRMZM2G339122), two EXPB3 transcripts (GRMZM2G094990, -1.95 log_2-fold; GRMZM2G176595), an EXPA7 (GRMZM2G445169) and two EXPB14 genes (GRMZM2G148485; GRMZM2G026956) were all down-regulated at Day 7. A putative polyphenol oxidase (PPO) family protein (AC209206.3_FG014) was expressed during early infection (24 hpi) but was repressed at Day 7 (Table 8).

The expression of hormone-based systemic signalling pathways: Numerous genes associated with hormone signalling were differentially expressed in inoculated kernels 24 hpi (Table 9). Several ethylene-associated genes, however, were down-regulated at 24 hpi, including an ethylene (ET) forming enzyme (GRMZM2G126732), two ET receptor genes (GRMZM2G077008; GRMZM2G318689, -1.94 log_2-fold), an ethylene-responsive transcription factor 1 (GRMZM2G085964, -2.19 log_2-fold) and an ethylene-responsive transcription factor 4 (GRMZM2G174347). An ethylene-responsive transcription factor 1 (GRMZM2G050851, 1.06 log_2-fold) was induced at the same time point. Gibberellin-encoding genes such as a gibberellic acid insensitive transcription factor (GRMZM2G144744), a gibberellic acid-insensitive phloem protein (GRMZM2G079470), a gibberellin C-20 oxidase 2 (GRMZM2G049418, -2.53 log_2-fold change; GRMZM2G121700), gibberellin-insensitive dwarf protein 1 (GRMZM2G079949) and a gibberellin-regulated protein (GRMZM2G068202) were all down-regulated 24 hpi. Additional hormone signalling pathways, such as auxin (indole acetic acid (IAA)) signalling with three transcripts encoding for indole-3-acetic acid-amino synthetase (GRMZM2G033359; GRMZM2G053338; GRMZM2G378106) and the jasmonate (JA) pathway with JA-induced protein genes (GRMZM2G172204; GRMZM2G112238), were repressed. An auxin response factor 1 gene (GRMZM2G137413, 1.93 log_2-fold) and a suppressor of auxin resistance1 (GRMZM2G167741, 2.25 log_2-fold) were strongly induced 24 hpi, whereas a chitinase gene (AC204711.3_FG002), which is a typical JA-associated gene, was down-regulated (Table 9).

A JA-induced protein was up-regulated at 48 hpi (GRMZM2G112238) and down-regulated at 72 hpi, while two chitinases (GRMZM2G453805; GRMZM2G053206) were down-regulated 48 hpi (Table 9). The ethylene-responsive transcription factor 1 (GRMZM2G050851), as well as a jasmonate-induced protein (GRMZM2G112238, -3.02 log_2-fold change), were repressed at 72 hpi while an ethylene-responsive transcription factor 4 (GRMZM2G174347), and an ethylene-responsive protein (GRMZM2G145974) as well as a gibberellin C-20 oxidase 1-B (GRMZM2G121700) were all induced. Two chitinases (GRMZM2G051943; GRMZM2G447795) were also induced at 72 hpi. At Day 7, two indole-3-acetic acid-amino synthetase genes (GRMZM2G033359; GRMZM2G053338) were

repressed while a putative auxin transport protein (GRMZM2G312110) was induced. A gibberellin 20 oxidase 1-B (GRMZM2G121700) as well a SAUR12-auxin-responsive SAUR family member (GRMZM2G475683, -2.81 log_2-fold) were also down-regulated (Table 9).

The expression of secondary metabolism-related genes: At 24 hpi, a chalcone flavonone isomerase (GRMZM2G175076), which functions in flavonoid synthesis, and two terpene synthase encoding genes (GRMZM2G127087; AC214360.3_FG001, 2.24 log_2-fold) were all induced (Table 10). Concurrently, three phenylalanine ammonia-lyase (PAL) genes GRMZM2G029048; GRMZM2G074604; GRMZM2G081582) were repressed. Additionally, the expression of three agmatine coumaroyltransferase genes (GRMZM2G013530; GRMZM2G066049; GRMZM2G066142), involved in the biosynthesis of hydroxycinnamic acid amides which plays a role in defence against pathogens, were reduced at 24 hpi when compared to the water-inoculated control. The terpene synthases (GRMZM2G127087, -3.44 log_2-fold; AC214360.3_FG001, -2.89 log_2-fold) were strongly down-regulated at 48 hpi, while a PAL gene (GRMZM2G081582) and a caffeic acid 3-O-methyltransferase gene (GRMZM5G814904) were induced at 72 hpi but repressed at Day 7. A single agmatine coumaroyltransferase gene (GRMZM2G013530) was down-regulated at Day 7, with no other secondary metabolism-related genes differentially expressed at 7 dai (Table 10).

***Fusarium verticillioides* quantification by quantitative real-time PCR (qRT-PCR)**

No significant differences in fungal target DNA could be determined in grain inoculated with *F. verticillioides* when compared to water-inoculated grain. The fungal target DNA, however, was slightly higher in inoculated grain at 24 hpi (0.006 ng uL^{-1}) when compared to the water control (0.005 ng uL^{-1}) and similar results were found at 48 hpi for inoculated (0.006 ng uL^{-1}) and water-inoculated grain (0.005 ng uL^{-1}). More fungal contamination was found in inoculated grain at 72 hpi (0.007 ng uL^{-1}) when compared to control kernels (0.005 ng uL^{-1}), 7 dai (0.006 ng uL^{-1}) and in 7 dai with water (0.005 ng uL^{-1}).

DISCUSSION

Maize ears responded to infection by *F. verticillioides* through a series of defence and metabolic pathways. Within 24 hours, PAMP recognition receptors (PRRs), characterised as LRRs, were strongly transcribed. The transcription of receptors stopped at 48 hpi, and was even down-regulated after 72 hpi, thereby indicating the ability of *F. verticillioides* to circumvent basal plant surveillance mechanisms in order to establish infection. Several types of receptor-like kinases (RLKs) were differentially expressed within 72 hours after chrysanthemum leaves were inoculated with *Alternaria tenuissima* Kunze (Wiltshire)

compared to water-inoculated controls (Li *et al*., 2014). Similarly, the expression of LRKs was regulated in lettuce 96 hpi when infected with *Botrytis cinerea* Pers. (De Cremer *et al*., 2013). Higher fragments per kilobase of exon model per million mapped reads (FPKM) values of RLKs-LLR were identified in the FER-resistant CO441 control kernels compared to FER-susceptible maize line CO354 (Lanubile *et al*., 2014). Specifically induced RLK-LRR in the susceptible (CO354) also showed higher FPKM values CO441 upon inoculation indicating a stronger pathogen induction in the resistant genotype (Lanubile *et al*., 2014). The down-regulation of receptors in comparison to water-inoculated control therefore appears to be induced by *F. verticillioides*.

After *F. verticillioides* was recognised by PRRs, signalling pathways were differentially regulated from 24 hpi to 7 dai. Several protein kinases (PKs) especially mitogen-associated protein kinases, serine/threonine-PKs and calcium-dependant PKs, were induced at 24 hpi which implies an early response to prevent *F. verticillioides* infection of maize kernels (Rodriguez *et al.*, 2010; Meng and Zhang, 2013; Lanubile *et al.*, 2014). Their expression, however, was repressed at 48 and 72 hpi with an induction observed at Day 7. The initial induction of PKs followed by the repression of these genes and PRRs, while *F. verticillioides* colonisation increased, suggests the ability of *F. verticillioides* to avoid the plant's defence system, thereby causing a delayed defence response. The down-regulation of GTP-binding proteins from 24 hpi to 72 hpi supports the association of G-proteins as signal molecules for defence responses in maize against *F. verticillioides* (Lanubile *et al.*, 2014). Notably, the induction of PKs and a G-protein coupled receptor at 7 dai, however, coincided with a reduction of fungal target DNA.

The transport of calcium ions across the plasma membrane of plant cells is one of the earliest cellular events following pathogen recognition (Atkinson *et al*., 1996; Jabs *et al*., 1997; Lecourieux *et al.*, 2006). In the current study, calcium homeostasis regulators were weakly induced (q-values: 0.001804–0.000526) with a single gene up-regulated (1.24 $\log_2$-fold; q-value: $1.4E^{-07}$) at 24 hpi. Calmodulin, along with other putative genes involved in calcium homeostasis, were repressed 24-72 hpi with the induction at 7 dai of a gene encoding calreticulin (GRMZM2G358059; q-value: $3.73E^{-05}$). These results demonstrate that calcium signalling was repressed and significantly affected by fungal colonisation. Reactive oxygen species (ROS), such as glutathione S-transferase (GST) (GST19) and peroxidase, were also transcribed after 24 hpi. GST is a widely recognised marker for ROS accumulation during plant defence (Marss, 1996; Lamb and Dixon, 1997; Dixon *et al.*, 2009), while peroxides are reactive compounds that pose an oxidation threat to cells when they accumulate to high concentrations (Dietz *et al*., 2006). Their build-up leads to redox signalling, which activated PR protein-encoding genes in the *F. verticillioides*-inoculated maize kernels at 72 hpi. PR proteins inhibit the proliferation of invading pathogens and

activate the systemic acquired resistance (SAR) response in plants (Ward *et al.*, 1991; Ryals *et al.,* 1996; Kitajima and Sato, 1999; Sekhon *et al.*, 2006). PR protein genes have reportedly been induced in both resistant and susceptible maize inbred lines in earlier studies (Murillo *et al.*, 1997; Murillo *et al.*, 2001; Lanubile *et al.*, 2010; 2012a; 2012b; 2014). The suppression of PR protein genes 7 dai with *F. verticillioides* may explain the increased colonisation of maize kernels with *F. verticillioides*.

Defence-related genes other than the PR proteins were also induced in the maize kernels following inoculation with *F. verticillioides*. These genes included a callose synthase enzyme, which was strongly induced (5.2 $\log_2$-fold; q-value: 0.00055664) at 24 hpi, along with a number of callose synthase genes and starch branching enzymes. Callose synthase enzymes synthesise callose for the reinforcement of cell walls at the site of infection (Chen and Kim, 2009). The starch branching enzymes were down-regulated 72 hpi, whereas expansin-encoding genes were induced. Expansins are known to have cell-wall loosening activity and are involved in cell expansion and other developmental events during which cell-wall modification occurs (Sampedro and Cosgrove, 2005).

Some defence-related genes were down-regulated following inoculation of maize kernels with *F. verticillioides.* For instance, a putative polyphenol oxidase (PPO) family protein gene was down-regulated at 24 hpi and remained down-regulated 7 days after infection. The overexpression of PPO is associated with resistance to plant pathogens of crops such as wheat, potato, sweet potato and tomato (Bashan *et al.*, 1987; Constabel *et al.*, 1995; Thipyapong *et al.*, 1995; Li and Steffens, 2002; Mohammadi and Kazemi, 2002, Ngadze *et al*., 2012). The suppression of PPO in this study may have allowed *F. verticillioides* to colonise maize kernels more efficiently.

JA and ET-signalling pathways are associated with PAMP-triggered plant immunity, whereas salicylic acid (SA)-mediated signalling pathways are associated with *R* gene-mediated plant resistance (Glazebrook, 2005). In this study, no transcriptional activation of SA-related genes was observed. Most of the ET-, gibberellin- and auxin-associated (indole acetic acid) genes were also down-regulated at 24 hpi. A JA-induced protein, though up-regulated (1.11 $\log_2$-fold; q-value: $9.359E^{-16}$) at 48 hpi was strongly down-regulated (-3.02 $\log_2$-fold; q-value: $4.724E^{-19}$) at 72 hpi with a number of indole acetic acid-related genes down-regulated at Day 7. These results are in contrast to Lanubile *et al.* (2014) who found that ET responsive proteins were up-regulated in both inoculated resistant and susceptible kernels, but mainly in the resistant line CO441, suggesting ET signalling involvement in *F. verticillioides*-maize pathosystem. The repression of these genes in the current study, however, may provide evidence of the importance of hormone signalling in providing plant defence to *F. verticillioides* and requires further confirmation. The auxin and SA pathways act in a mutually antagonistic manner during plant defence (Wang *et al.*, 2007; Kazan and

Manners, 2009) and the regulation of auxin-associated genes in this study provides support for a PAMP-triggered immunity in maize plants following *F. verticillioides* infection. The induction of a probable indole-3-acetic acid amido synthetase (1.37 log_2-fold; q-value: $1.0419E^{-06}$) at 7 dai provides evidence of an auxin hormone signalling event following the initial infection by *F. verticillioides*.

An understanding of the genetic mechanisms underpinning resistance to *F. verticillioides* is required for the development of resistant maize breeding lines and cultivars. The current study provided some knowledge on the transcriptional changes that occur in maize following infection with *F. verticillioides*. It further identified several genes which potentially influence *F. verticillioides* infection of maize, including defence-related genes such as PRRs, signalling molecules (protein kinases, calcium-dependant molecules, GTP-signalling and redox-associated molecules), PR protein-coding genes and those involved in secondary hormone signalling (auxins). Their role should be verified by gene knock-out, gene silencing or over-expression in model plants. The suppression of most PR genes at 24 hpi, despite the significant induction of zeamatin and other PR genes (PR6, PR1 and defensin-like protein) suggests a delayed and/or ineffective basal defence response to infection by *F. verticillioides*.

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Table 1. Number of raw and clean reads, Phred scores and guanine/cytosine content following RNA sequencing of maize inoculated with *Fusarium verticillioides*.

Time point[1]	Raw reads	Clean reads	Q20 (%)[2]	Q30 (%)[3]	GC Content[4]
Time 0	9 085 830	9 064 061	99.0	96.9	54.1
T1, Control	7 572 305	7 537 489	98.8	96.2	55.2
T1, Inoculated	8 883 239	8 854 206	98.9	96.7	52.1
T2, Control	10 185 317	10 159 178	99.0	96.9	54.0
T2, Inoculated	9 218 945	9 190 666	99.0	96.8	55.1
T3, Control	10 891 312	10 862 340	98.9	96.5	53.0
T3, Inoculated	8 870 637	8 819 580	98.8	96.2	55.2
T4, Control	9 854 620	9 813 899	98.9	96.6	56.2
T4, Inoculated	8 384 444	8 348 944	98.9	96.6	54.6

[1]T1: 24 hours after inoculation (hai), T2: 48 hai, T3: 72 hai and T4: 7 days after inoculation

[2]The percentage of the base number of Phred score greater than 20 (Q20) accounting for the total number of bases

[3] The percentage of the base number of Phred score greater than 30 (Q30) accounting for the total number of bases

[4]The percentage of the bases G and C accounting for the total number of bases

Table 2. Mapping of clean reads to the reference genome of *Zea mays* following RNA sequencing of maize inoculated with *Fusarium verticillioides*.

Time point[1]	Total reads[2]	Total mapped[3]	Multiple mapped[4]	Uniquely mapped[5]
0	9 064 061	8 216 464 **(90.7%)**	1 388 785 **(15.3%)**	6 827 679 **(75.3%)**
T1, Control	7 537 489	6 761 323 **(89.7%)**	915 230 **(12.1%)**	5 846 093 **(77.6%)**
T1, Inoculated	8 854 206	7 988 083 **(90.2%)**	1 568 430 **(17.7%)**	6 419 653 **(72.5%)**
T2, Control	10 159 178	9 170 639 **(90.3%)**	1 494 195 **(14.7%)**	7 676 444 **(75.6%)**
T2, Inoculated	9 190 666	8 297 821 **(90.3%)**	1 145 880 **(12.5%)**	7 151 941 **(77.8%)**
T3, Control	10 862 340	9 880 465 **(91.0%)**	1 778 455 **(16.4%)**	8 102 010 **(74.6%)**
T3, Inoculated	8 819 580	7 929 913 **(89.9%)**	1 130 917 **(12.8%)**	6 798 996 **(77.1%)**
T4, Control	9 813 899	8 780 404 **(89.5%)**	1 174 218 **(12.0%)**	7 606 186 **(77.5%)**
T4, Inoculated	8 348 944	7 490 994 **(89.7%)**	1 177 354 **(14.1%)**	6 313 640 **(75.6%)**

[1]T1: 24 hours after inoculation (hai), T2: 48 hai, T3: 72 hai and T4: 7 days after inoculation

[2]Total reads=Count number of filtered reads (clean data)

[3]Total mapped=Total number of reads that could be mapped to the genome. In general, this digit should be larger than 70% when there is no contamination and the reference genome is wisely chosen

[4]Multiple mapped=Count number of reads that could be mapped to multiple sites in the reference genome, which should be less than 10% in commonplace

[5]Uniquely mapped=Count number of reads that can be uniquely mapped to the reference genome

Table 3. Differentially expressed genes associated with pathogen recognition receptors in maize kernels following inoculation with *Fusarium verticillioides*.

Day(s)	Gene ID	Sequence description	Molecular function and description	Putative annotation (BLAST SwissProt)	Inoculated	Control	Fold change	q value
1	GRMZM5G812774	BES transcription factor%3B Brassinazole-resistant 1 protein%3B MYBGA transcription factor%3B Uncharacterised protein	#N/A	sp|Q9ZV88|BEH4_ARATH BES1/BZR1 homolog protein 4 OS=Arabidopsis thaliana GN=BEH4 PE=1 SV=1//7.42319e-20	17.58	17.58	-1.95	3.809E-06
1	GRMZM2G006080	Putative receptor-like protein kinase family protein	GO:0004672//GO:0005524 protein kinase activity//ATP binding	sp|Q9SCZ4|FERON_ARATH Receptor-like protein kinase FERONIA OS=Arabidopsis thaliana GN=FER PE=1 SV=1//0	36.28	36.28	-1.13	0.0032119
1	GRMZM2G011526	#N/A	GO:0005515//GO:0005524//GO:0004672 protein binding//ATP binding//protein kinase activity	sp|C0LGE0|Y1765_ARATH Probable LRR receptor-like serine/threonine-protein kinase At1g07650 OS=Arabidopsis thaliana GN=At1g07650 PE=1 SV=1//0	24.93	24.93	2.93	0.0004151
1	GRMZM2G126858	Putative leucine-rich repeat receptor-like protein kinase family protein	GO:0005524//GO:0005515//GO:0004672 ATP binding//protein binding//protein kinase activity	sp|C0LGH2|Y1561_ARATH Probable LRR receptor-like serine/threonine-protein kinase At1g56130 OS=Arabidopsis thaliana GN=At1g56130 PE=1 SV=2//1.15066e-07	22.26	22.26	3.58	0.0002556
1	GRMZM2G176922	#N/A	GO:0005515//GO:0005524//GO:0004672 protein binding//ATP binding//protein kinase activity	sp|C0LGT1|Y5129_ARATH Probable LRR receptor-like serine/threonine-protein kinase At5g10290 OS=Arabidopsis thaliana GN=At5g10290 PE=1 SV=1//1.23511e-43	40.28	40.28	2.19	8.445E-05
3	GRMZM2G176922	RPK1_IPONI Receptor-like protein kinase precursor	GO:0005515//GO:0005524//GO:0004672 protein binding//ATP binding//protein kinase activity	sp|C0LGT1|Y5129_ARATH Probable LRR receptor-like serine/threonine-protein kinase At5g10290 OS=Arabidopsis thaliana GN=At5g10290 PE=1 SV=1//1.23511e-43	13.19	13.19	-1.95	3.83E-05

Table 4. Differentially expressed genes associated with putative protein kinases in maize kernels following inoculation with *Fusarium verticillioides*.

Day(s)	Gene ID	Sequence description	Molecular function and description	Putative annotation (BLAST SwissProt)	Inoculated	Control	Fold change	q value
1	GRMZM5G897067	#N/A	GO:0030554 adenyl nucleotide binding	sp|Q9XI37|PV42A_ARATH SNF1-related protein kinase regulatory subunit gamma-like PV42a OS=Arabidopsis thaliana GN=PV42A PE=1 SV=1//1.88899e-94	47.41	126.91	-1.42	3.65E-07
1	GRMZM2G025242	MAPK activating protein%3B Uncharacterised protein	GO:0015002 heme-copper terminal oxidase activity	sp|Q54PI4|T1841_DICDI Transmembrane protein 184 homolog DDB_G0284525 OS=Dictyostelium discoideum GN=tmem184A PE=3 SV=1//9.85237e-19	108.84	234.72	-1.11	8.35E-09
1	GRMZM2G093316	Putative MAPKKK family protein kinase isoform 1%3B Putative MAPKKK family protein kinase isoform 2	GO:0005524//GO:0004672 ATP binding//protein kinase activity	sp|Q9FZ36|M3K2_ARATH Mitogen-activated protein kinase kinase kinase 2 OS=Arabidopsis thaliana GN=ANP2 PE=2 SV=1//7.47446e-06	86.36	41.91	1.04	0.000949
1	GRMZM2G092959	Uncharacterised protein	#N/A	-//-	88.58	36.79	1.27	4.67E-05
1	GRMZM2G075683	Uncharacterised protein	GO:0005524//GO:0004672// GO:0016773 ATP binding//protein kinase activity//phosphotransferase activity, alcohol group as acceptor	sp|P42158|KC1D_ARATH Casein kinase I isoform delta-like OS=Arabidopsis thaliana GN=At4g26100 PE=2 SV=2//4.48204e-161	67.44	27.24	1.31	0.000446
1	GRMZM2G312970	Uncharacterised protein	GO:0004672//GO:0005524 protein kinase activity//ATP binding	sp|Q65X23|WNK2_ORYSJ Probable serine/threonine-protein kinase WNK2 OS=Oryza sativa subsp. japonica GN=WNK2 PE=2 SV=1//2.51786e-31	53.19	20.49	1.38	0.001635
1	GRMZM2G114162	#N/A	GO:0008565//GO:0016773 protein transporter activity//phosphotransferase activity, alcohol group as acceptor	sp|Q9SXA1|P4KA1_ARATH Phosphatidylinositol 4-kinase alpha OS=Arabidopsis thaliana GN=PI4KALPHA1 PE=1 SV=2//0	54.08	18.63	1.54	0.000407

1	GRMZM2G322846	#N/A	GO:0005515 protein binding	-//-	56.31	19.09	1.56	0.000231
1	GRMZM2G311051	Uncharacterised protein	#N/A	-//-	97.71	32.83	1.57	1.45E-07
1	GRMZM2G103721	Uncharacterised protein	GO:0005515//GO:0016773//GO:0005198//GO:0004867 protein binding//phosphotransferase activity, alcohol group as acceptor//structural molecule activity//serine-type endopeptidase inhibitor activity	sp|P42347|PI3K1_SOYBN Phosphatidylinositol 3-kinase, root isoform OS=Glycine max PE=2 SV=1//0	60.98	19.33	1.66	4.33E-05
1	GRMZM2G147811	#N/A	GO:0046873//GO:0003779//GO:0005524//GO:0004013//GO:0004672 metal ion transmembrane transporter activity//actin binding//ATP binding// Adenosyl homocysteinase activity//protein kinase activity	sp|Q13523|PRP4B_HUMAN Serine/threonine-protein kinase PRP4 homolog OS=Homo sapiens GN=PRPF4B PE=1 SV=3//1.76365e-08	44.07	13.04	1.76	0.000504
1	AC218998.2_FG007	Uncharacterised protein	GO:0005524//GO:0004672 ATP binding//protein kinase activity	sp|P83304|LEC_PARPC Mannose/glucose-specific lectin (Fragment) OS=Parkia platycephala PE=1 SV=1//6.11714e-24	36.72	9.31	1.98	0.000642
1	GRMZM2G151236	Uncharacterised protein	GO:0003677//GO:0005524//GO:0008430//GO:0003779//GO:0046873//GO:0004672 DNA binding//ATP binding//selenium binding//actin binding//metal ion transmembrane transporter activity//protein kinase activity	sp|Q5RKH1|PRP4B_RAT Serine/threonine-protein kinase PRP4 homolog OS=Rattus norvegicus GN=Prpf4b PE=2 SV=1//4.64926e-09	42.51	9.78	2.12	7.14E-05
1	GRMZM2G094039	Uncharacterised protein	GO:0003735//GO:0005524//GO:0016773//GO:0004672 structural constituent of ribosome//ATP binding//phosphotransferase activity, alcohol group as acceptor//protein kinase activity	sp|P51568|AFC3_ARATH Serine/threonine-protein kinase AFC3 OS=Arabidopsis thaliana GN=AFC3 PE=2 SV=2//6.71392e-09	22.92	3.49	2.71	0.001512

2	GRMZM2G142019	Putative DUF869 domain containing family protein	GO:0005096//GO:0004674 GTPase activator activity//protein serine/threonine kinase activity	sp|Q8LLE5|FPP_SOLLC Filament-like plant protein (Fragment) OS=Solanum lycopersicum GN=FPP PE=1 SV=1//1.60505e-63	16.26	44.82	-1.46	0.00378
2	AC205419.3_FG001	Putative DUF869 domain containing family protein	#N/A	sp|Q0WSY2|FPP4_ARATH Filament-like plant protein 4 OS=Arabidopsis thaliana GN=FPP4 PE=2 SV=1//3.76062e-171	36.04	87.2	-1.27	3.65E-05
2	GRMZM2G093316	Putative MAPKKK family protein kinase isoform 1%3B Putative MAPKKK family protein kinase isoform 2	GO:0005524//GO:0004672 ATP binding//protein kinase activity	sp|Q9FZ36|M3K2_ARATH Mitogen-activated protein kinase kinase kinase 2 OS=Arabidopsis thaliana GN=ANP2 PE=2 SV=1//7.47446e-06	38.79	91.91	-1.24	2.88E-05
3	GRMZM2G142019	Putative DUF869 domain containing family protein	GO:0005096//GO:0004674 GTPase activator activity//protein serine/threonine kinase activity	sp|Q8LLE5|FPP_SOLLC Filament-like plant protein (Fragment) OS=Solanum lycopersicum GN=FPP PE=1 SV=1//1.60505e-63	10.19	42.14	-2.05	0.000172
3	GRMZM2G142019	Putative DUF869 domain containing family protein	GO:0005096//GO:0004674 GTPase activator activity//protein serine/threonine kinase activity	sp|Q8LLE5|FPP_SOLLC Filament-like plant protein (Fragment) OS=Solanum lycopersicum GN=FPP PE=1 SV=1//1.60505e-63	10.19	42.14	-2.05	0.000172
3	GRMZM2G147811	#N/A	GO:0046873//GO:0003779// GO:0005524//GO:0004013// GO:0004672 metal ion transmembrane transporter activity//actin binding//ATP binding//adenosylhomocysteinase activity//protein kinase activity	sp|Q13523|PRP4B_HUMAN Serine/threonine-protein kinase PRP4 homolog OS=Homo sapiens GN=PRPF4B PE=1 SV=3//1.76365e-08	11.99	44.66	-1.9	0.000252
3	GRMZM2G151236	Uncharacterised protein	GO:0003677//GO:0005524// GO:0008430//GO:0003779// GO:0046873//GO:0004672 DNA binding//ATP binding//selenium binding//actin binding//metal ion transmembrane transporter activity//protein	sp|Q5RKH1|PRP4B_RAT Serine/threonine-protein kinase PRP4 homolog OS=Rattus norvegicus GN=Prpf4b PE=2 SV=1//4.64926e-09	13.58	50.56	-1.9	6.75E-05

			kinase activity					
3	GRMZM2G103721	Uncharacterised protein	GO:0005515//GO:0016773//GO:0005198//GO:0004867 protein binding//phosphotransferase activity, alcohol group as acceptor//structural molecule activity//serine-type endopeptidase inhibitor activity	sp|P42347|PI3K1_SOYBN Phosphatidylinositol 3-kinase, root isoform OS=Glycine max PE=2 SV=1//0	20.18	55.45	-1.46	0.000894
3	GRMZM2G093316	Putative MAPKKK family protein kinase isoform 1%3B Putative MAPKKK family protein kinase isoform 2	GO:0005524//GO:0004672 ATP binding//protein kinase activity	sp|Q9FZ36|M3K2_ARATH Mitogen-activated protein kinase kinase kinase 2 OS=Arabidopsis thaliana GN=ANP2 PE=2 SV=1//7.47446e-06	52.94	137.87	-1.38	9.38E-09
3	AC205419.3_FG001	Putative DUF869 domain containing family protein	#N/A	sp|Q0WSY2|FPP4_ARATH Filament-like plant protein 4 OS=Arabidopsis thaliana GN=FPP4 PE=2 SV=1//3.76062e-171	35.16	88.49	-1.33	2.81E-05
3	AC205419.3_FG001	Putative DUF869 domain containing family protein	#N/A	sp|Q0WSY2|FPP4_ARATH Filament-like plant protein 4 OS=Arabidopsis thaliana GN=FPP4 PE=2 SV=1//3.76062e-171	35.16	88.49	-1.33	2.81E-05
3	GRMZM2G114162	#N/A	GO:0008565//GO:0016773 protein transporter activity//phosphotransferase activity, alcohol group as acceptor	sp|Q9SXA1|P4KA1_ARATH Phosphatidylinositol 4-kinase alpha OS=Arabidopsis thaliana GN=PI4KALPHA1 PE=1 SV=2//0	28.97	67.08	-1.21	0.001693
3	GRMZM5G897067	#N/A	GO:0030554 adenyl nucleotide binding	sp|Q9XI37|PV42A_ARATH SNF1-related protein kinase regulatory subunit gamma-like PV42a OS=Arabidopsis thaliana GN=PV42A PE=1 SV=1//1.88899e-94	138.04	67.42	1.03	0.000182

7	GRMZM2G032852	Uncharacterised protein	GO:0005509//GO:0005524//GO:0000166//GO:0004812 calcium ion binding//ATP binding//nucleotide binding//aminoacyl-tRNA ligase activity	sp|P53684|CDPKB_ORYSJ Calcium-dependent protein kinase isoform 11 OS=Oryza sativa subsp. japonica GN=CPK11 PE=2 SV=2//7.57169e-65	82.13	40.6	1.02	0.001011
7	GRMZM2G153722	Putative 1-phosphatidylinositol-4-phosphate 5-kinase/ zinc ion binding family	GO:0008270//GO:0046872//GO:0005524//GO:0017137//GO:0016307 zinc ion binding//metal ion binding//ATP binding//Rab GTPase binding//phosphatidylinositol phosphate kinase activity	sp|P34756|FAB1_YEAST 1-phosphatidylinositol 3-phosphate 5-kinase FAB1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=FAB1 PE=1 SV=3//6.53707e-09	115.6	53.75	1.1	5.68E-06
7	GRMZM2G093316	Putative MAPKKK family protein kinase isoform 1%3B Putative MAPKKK family protein kinase isoform 2	GO:0005524//GO:0004672 ATP binding//protein kinase activity	sp|Q9FZ36|M3K2_ARATH Mitogen-activated protein kinase kinase kinase 2 OS=Arabidopsis thaliana GN=ANP2 PE=2 SV=1//7.47446e-06	95.85	32.59	1.56	7.6E-08

Table 5. Differentially expressed genes associated with calcium homeostasis in maize kernels following inoculation with *Fusarium verticillioides*.

Day(s)	Gene ID	Sequence description	Molecular function and description	Putative annotation (BLAST SwissProt)	Inoculated	Control	Fold change	q value
1	GRMZM2G152891	Calmodulin%3B Uncharacterised protein	GO:0005509//GO:0003899 calcium ion binding//DNA-directed RNA polymerase activity	sp|P27164|CALM3_PETHY Calmodulin-related protein OS=Petunia hybrida GN=CAM53 PE=2 SV=2//6.20982e-105	17.81	51.93	-1.54	0.001868
1	GRMZM2G114775	Putative uncharacterised protein	GO:0005219//GO:0008270//GO:0003700//GO:0043565//GO:0031683//GO:0003924//GO:0004871//GO:0019001 ryanodine-sensitive calcium-release channel activity//zinc ion binding//sequence-specific DNA binding transcription factor activity//sequence-specific DNA binding//G-protein beta/gamma-subunit complex binding//GTPase activity//signal transducer activity//guanyl nucleotide binding	sp|Q9FJ10|GAT16_ARATH GATA transcription factor 16 OS=Arabidopsis thaliana GN=GATA16 PE=2 SV=1//1.89702e-14	58.31	140.65	-1.27	1.28E-06
1	GRMZM2G010868	Non-specific lipid-transfer protein	#N/A	sp|Q43194|NLTP2_SORBI Non-specific lipid-transfer protein 2 OS=Sorghum bicolor GN=LTP2 PE=3 SV=1//5.34955e-42	130.2	267.79	-1.04	5.47E-09
1	GRMZM2G091189	Uncharacterised protein	#N/A	sp|O65282|CH10C_ARATH 20 kDa chaperonin, chloroplastic OS=Arabidopsis thaliana GN=CPN21 PE=1 SV=2//7.43708e-10	123.97	250.09	-1.01	5.57E-08
1	GRMZM2G358059	Calreticulin	GO:0005509//GO:0051082 calcium ion binding//unfolded protein binding	sp|Q9SP22|CALR_MAIZE Calreticulin OS=Zea mays GN=CRT PE=2 SV=1//8.20812e-06	137.55	58.22	1.24	1.4E-07

1	GRMZM2G305115	Uncharacterised protein	GO:0051082//GO:0005509 unfolded protein binding//calcium ion binding	sp|O04153|CALR3_ARATH Calreticulin-3 OS=Arabidopsis thaliana GN=CRT3 PE=2 SV=2//9.48402e-172	44.74	16.53	1.44	0.003565
1	GRMZM2G052875	Uncharacterised protein	GO:0005515 protein binding	sp|P14314|GLU2B_HUMAN Glucosidase 2 subunit beta OS=Homo sapiens GN=PRKCSH PE=1 SV=2//5.96873e-22	45.85	16.07	1.51	0.001804
1	GRMZM2G074687	#N/A	GO:0008270//GO:0003700//GO:0051082//GO:0016987//GO:0005509//GO:0003677 zinc ion binding//sequence-specific DNA binding transcription factor activity//unfolded protein binding//sigma factor activity//calcium ion binding//DNA binding	sp|Q9SLY8|CALR_ORYSJ Calreticulin OS=Oryza sativa subsp. japonica GN=Os07g0246200 PE=1 SV=2//3.7106e-11	33.83	10.48	1.69	0.00499
1	GRMZM2G167982	#N/A	GO:0016757//GO:0051082 transferase activity, transferring glycosyl groups//unfolded protein binding	-//-	24.48	3.26	2.91	0.000526
2	GRMZM2G305115	Uncharacterised protein	GO:0051082//GO:0005509 unfolded protein binding//calcium ion binding	sp|O04153|CALR3_ARATH Calreticulin-3 OS=Arabidopsis thaliana GN=CRT3 PE=2 SV=2//9.48402e-172	11.17	40.46	-1.86	0.000694
2	GRMZM2G358059	Calreticulin	GO:0005509//GO:0051082 calcium ion binding//unfolded protein binding	sp|Q9SP22|CALR_MAIZE Calreticulin OS=Zea mays GN=CRT PE=2 SV=1//8.20812e-06	39.77	93.31	-1.23	2.9E-05
2	GRMZM2G145905	Uncharacterised protein	GO:0003700//GO:0003676 sequence-specific DNA binding transcription factor activity//nucleic acid binding	sp|Q5ZK33|LETM1_CHICK LETM1 and EF-hand domain-containing protein 1, mitochondrial OS=Gallus gallus GN=LETM1 PE=2 SV=1//7.95329e-40	51.91	113.54	-1.13	1.09E-05

3	GRMZM2G305115	Uncharacterised protein	GO:0051082//GO:0005509 unfolded protein binding//calcium ion binding	sp|O04153|CALR3_ARATH Calreticulin-3 OS=Arabidopsis thaliana GN=CRT3 PE=2 SV=2//9.48402e-172	11.99	42.98	-1.84	0.000518
3	GRMZM2G052875	Uncharacterised protein	GO:0005515 protein binding	sp|P14314|GLU2B_HUMAN Glucosidase 2 subunit beta OS=Homo sapiens GN=PRKCSH PE=1 SV=2//5.96873e-22	17.38	47.7	-1.46	0.002991
3	GRMZM2G163494	Component of high affinity nitrate transporter	#N/A	sp|Q9FGS5|NRT31_ARATH High-affinity nitrate transporter 3.1 OS=Arabidopsis thaliana GN=NRT3.1 PE=1 SV=1//9.34362e-25	27.37	4.21	2.7	0.001145
7	GRMZM2G358059	Calreticulin	GO:0005509//GO:0051082 calcium ion binding//unfolded protein binding	sp|Q9SP22|CALR_MAIZE Calreticulin OS=Zea mays GN=CRT PE=2 SV=1//8.20812e-06	101.46	47.46	1.1	3.73E-05
7	GRMZM2G391042	Uncharacterised protein	GO:0046872//GO:0004675//GO:0000166//GO:0005524//GO:0005516 metal ion binding//transmembrane receptor protein serine/threonine kinase activity//nucleotide binding//ATP binding//calmodulin binding	sp|Q9LF79|ACA8_ARATH Calcium-transporting ATPase 8, plasma membrane-type OS=Arabidopsis thaliana GN=ACA8 PE=1 SV=1//1.00547e-35	35.55	10.48	1.76	0.002551

Table 6. Differentially expressed genes associated with GTP- and redox signalling in maize kernels following inoculation with *Fusarium verticillioides*.

Day(s)	Gene ID	Sequence description	Molecular function and description	Putative annotation (BLAST SwissProt)	Inoculated	Control	Fold change	q value
1	GRMZM2G172932	Uncharacterised protein	GO:0005525//GO:0003924 GTP binding//GTPase activity	sp|Q9ZPN9|TBB2_ELEIN Tubulin beta-2 chain OS=Eleusine indica GN=TUBB2 PE=2 SV=1//3.4176e-40	25.37	67.06	-1.4	0.000864
1	GRMZM2G071790	Tubulin beta-6 chain	GO:0005525//GO:0003924 GTP binding//GTPase activity	sp|P25862|TBB1_AVESA Tubulin beta-1 chain (Fragment) OS=Avena sativa GN=TUBB1 PE=2 SV=1//5.85354e-39	53.42	134.83	-1.34	6.77E-07
1	GRMZM2G365862	#N/A	GO:0005525 GTP binding	-//-	22.03	0	4.56	7.17E-05
1	GRMZM2G044383	Glutathione S-transferase GST 30%3B Uncharacterised protein	GO:0005515 protein binding	sp|Q06398|GSTU6_ORYSJ Probable glutathione S-transferase GSTU6 OS=Oryza sativa subsp. japonica GN=GSTU6 PE=2 SV=2//1.67225e-102	193.19	682.98	-1.82	4.92E-58
1	GRMZM2G129357	Uncharacterised protein	GO:0005515 protein binding	sp|Q8L7C9|GSTUK_ARATH Glutathione S-transferase U20 OS=Arabidopsis thaliana GN=GSTU20 PE=1 SV=1//1.38259e-23	10.68	36.56	-1.77	0.004873
1	GRMZM2G114988	Putative cytochrome P450 superfamily protein	GO:0070008//GO:0003968//GO:0004252//GO:0016817//GO:0016705//GO:0004197//GO:0005506//GO:0017111//GO:0020037 serine-type exopeptidase activity//RNA-directed RNA polymerase activity//serine-type endopeptidase activity//hydrolase activity, acting on acid anhydrides//oxidoreductase activity, acting on paired donors, with	sp|P93147|C81E1_GLYEC Isoflavone 2'-hydroxylase OS=Glycyrrhiza echinata GN=CYP81E1 PE=1 SV=2//1.60359e-105	73.89	207.48	-1.49	9.85E-13

			incorporation or reduction of molecular oxygen//cysteine-type endopeptidase activity//iron ion binding//nucleoside-triphosphatase activity//heme binding						
1	GRMZM2G129761	1-Cys peroxiredoxin PER1	GO:0016209//GO:0016491//GO:0004930//GO:0051920 antioxidant activity//oxidoreductase activity//G-protein coupled receptor activity//peroxiredoxin activity	sp|A2SZW8|REHY_MAIZE 1-Cys peroxiredoxin PER1 OS=Zea mays GN=PER1 PE=2 SV=1//2.11976e-154	902.07	2500.46	-1.47	4.2E-155	
1	GRMZM2G135536	Putative cytochrome P450 superfamily protein%3B Uncharacterised protein	GO:0020037//GO:0016705//GO:0005506 heme binding//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen//iron ion binding	sp|Q9SRQ1|C89A9_ARATH Cytochrome P450 89A9 OS=Arabidopsis thaliana GN=CYP89A9 PE=2 SV=1//1.04353e-101	21.37	57.28	-1.42	0.002324	
1	GRMZM2G067225	Putative cytochrome P450 superfamily protein%3B Uncharacterised protein	GO:0016705//GO:0020037//GO:0005506 oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen//heme binding//iron ion binding	sp|Q7XYS3|C74A2_ORYSJ Allene oxide synthase 2 OS=Oryza sativa subsp. japonica GN=CYP74A2 PE=2 SV=2//0	129.98	306.44	-1.24	6.26E-14	
1	GRMZM2G139874	Putative cytochrome P450 superfamily protein%3B Uncharacterised protein	GO:0005506//GO:0016705//GO:0020037 iron ion binding//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen//heme binding	sp|Q43054|TCMO_POPKI Trans-cinnamate 4-monooxygenase OS=Populus kitakamiensis GN=CYP73A16 PE=2 SV=1//1.0834e-07	49.19	114.8	-1.22	4.33E-05	

1	GRMZM2G036921	Peroxiredoxin-5	GO:0016491//GO:0016209 oxidoreductase activity//antioxidant activity	sp|Q69TY4|PR2E1_ORYSJ Peroxiredoxin-2E-1, chloroplastic OS=Oryza sativa subsp. japonica GN=PRXIIE-1 PE=2 SV=1//1.15423e-98	84.58	169.76	-1.01	2.35E-05
1	GRMZM2G154828	Uncharacterised protein	GO:0005506//GO:0016705//GO:0020037 iron ion binding//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen//heme binding	sp|P93147|C81E1_GLYEC Isoflavone 2'-hydroxylase OS=Glycyrrhiza echinata GN=CYP81E1 PE=1 SV=2//5.07788e-127	75.67	30.27	1.32	0.00013
1	GRMZM2G169201	#N/A	GO:0016866//GO:0016491 intramolecular transferase activity//oxidoreductase activity	sp|O81210|RBOHC_ARATH Respiratory burst oxidase homolog protein C OS=Arabidopsis thaliana GN=RBOHC PE=2 SV=2//3.56757e-112	128.2	50.07	1.36	4.66E-08
1	GRMZM2G154870	#N/A	GO:0020037//GO:0016705//GO:0005506 heme binding//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen//iron ion binding	sp|P93147|C81E1_GLYEC Isoflavone 2'-hydroxylase OS=Glycyrrhiza echinata GN=CYP81E1 PE=1 SV=2//1.49603e-116	29.82	6.29	2.25	0.000996
2	GRMZM2G028821	Glutathione S-transferase GSTU6	GO:0005515 protein binding	sp|P32111|GSTX1_SOLTU Probable glutathione S-transferase OS=Solanum tuberosum GN=PRP1 PE=2 SV=1//3.53727e-41	12.34	61.74	-2.32	6.3E-08
2	GRMZM2G159744	Uncharacterised protein	GO:0015078 hydrogen ion transmembrane transporter activity	-//-	18.22	67.49	-1.89	8.98E-07

2	GRMZM2G114988	Putative cytochrome P450 superfamily protein	GO:0070008//GO:0003968//GO:0004252//GO:0016817//GO:0016705//GO:0004197//GO:0005506//GO:0017111//GO:0020037 serine-type exopeptidase activity//RNA-directed RNA polymerase activity//serine-type endopeptidase activity//hydrolase activity, acting on acid anhydrides//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen//cysteine-type endopeptidase activity//iron ion binding//nucleoside-triphosphatase activity//heme binding	sp|P93147|C81E1_GLYEC Isoflavone 2'-hydroxylase OS=Glycyrrhiza echinata GN=CYP81E1 PE=1 SV=2//1.60359e-105	208.03	97.32	1.1	1.9E-06
2	GRMZM2G044383	Glutathione S-transferase GST 30%3B Uncharacterised protein	GO:0005515 protein binding	sp|Q06398|GSTU6_ORYSJ Probable glutathione S-transferase GSTU6 OS=Oryza sativa subsp. japonica GN=GSTU6 PE=2 SV=2//1.67225e-102	803.93	366.42	1.13	3.95E-28
3	GRMZM2G427424	#N/A	GO:0003924//GO:0005525//GO:0005515//GO:0004930 GTPase activity//GTP binding//protein binding//G-protein coupled receptor activity	sp|Q08BT5|K1468_XENTR LisH domain and HEAT repeat-containing protein KIAA1468 homolog OS=Xenopus tropicalis PE=2 SV=1//3.26522e-48	10.59	36.07	-1.77	0.003294
3	GRMZM2G147966	#N/A	GO:0003677//GO:0004601//GO:0005509//GO:0050664 DNA binding//peroxidase activity//calcium ion binding//oxidoreductase	sp|O81211|RBOHE_ARATH Respiratory burst oxidase homolog protein E OS=Arabidopsis thaliana GN=RBOHE PE=2 SV=2//4.72529e-78	30.57	85.62	-1.49	5.59E-06

			activity, acting on NADH or NADPH, oxygen as acceptor					
3	GRMZM2G157462	Dynamin-2A%3B Uncharacterised protein	GO:0005525//GO:0003924 GTP binding//GTPase activity	sp|Q9LQ55|DRP2B_ARATH Dynamin-2B OS=Arabidopsis thaliana GN=DRP2B PE=1 SV=2//0	46.75	106.52	-1.19	2.25E-05
3	GRMZM2G028313	Putative translation elongation/initiation factor family protein	GO:0005525//GO:0003924 GTP binding//GTPase activity	sp|P15170|ERF3A_HUMAN Eukaryotic peptide chain release factor GTP-binding subunit ERF3A OS=Homo sapiens GN=GSPT1 PE=1 SV=1//4.54367e-159	54.94	113.94	-1.05	8.96E-05
3	GRMZM2G114988	Putative cytochrome P450 superfamily protein	GO:0070008//GO:0003968//GO:0004252//GO:0016817//GO:0016705//GO:0004197//GO:0005506//GO:0017111//GO:0020037 serine-type exopeptidase activity//RNA-directed RNA polymerase activity//serine-type endopeptidase activity//hydrolase activity, acting on acid anhydrides//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen//cysteine-type endopeptidase activity//iron ion binding//nucleoside-triphosphatase activity//heme binding	sp|P93147|C81E1_GLYEC Isoflavone 2'-hydroxylase OS=Glycyrrhiza echinata GN=CYP81E1 PE=1 SV=2//1.60359e-105	166.61	82.76	1.01	3.53E-05

3	GRMZM2G335618	Glutathione S-transferase GST 19%3B Uncharacterised protein	GO:0005515 protein binding	sp|Q10CE7|GSTU1_ORYSJ Probable glutathione S-transferase GSTU1 OS=Oryza sativa subsp. japonica GN=GSTU1 PE=1 SV=1//2.59432e-73	201.57	93.88	1.1	2.39E-07
3	GRMZM2G044383	Glutathione S-transferase GST 30%3B Uncharacterised protein	GO:0005515 protein binding	sp|Q06398|GSTU6_ORYSJ Probable glutathione S-transferase GSTU6 OS=Oryza sativa subsp. japonica GN=GSTU6 PE=2 SV=2//1.67225e-102	606.32	281.81	1.11	6.98E-23
3	GRMZM2G036921	Peroxiredoxin-5	GO:0016491//GO:0016209 oxidoreductase activity//antioxidant activity	sp|Q69TY4|PR2E1_ORYSJ Peroxiredoxin-2E-1, chloroplastic OS=Oryza sativa subsp. japonica GN=PRXIIE-1 PE=2 SV=1//1.15423e-98	141.84	63.2	1.17	1.16E-05
3	GRMZM2G057086	Uncharacterised protein	GO:0005506//GO:0020037//GO:0016705 iron ion binding//heme binding//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	sp|Q9LZ31|C77A4_ARATH Cytochrome P450 77A4 OS=Arabidopsis thaliana GN=CYP77A4 PE=2 SV=1//0	106.88	45.17	1.24	9.6E-05
3	GRMZM2G129761	1-Cys peroxiredoxin PER1	GO:0016209//GO:0016491//GO:0004930//GO:0051920 antioxidant activity//oxidoreductase activity//G-protein coupled receptor activity//peroxiredoxin activity	sp|A2SZW8|REHY_MAIZE 1-Cys peroxiredoxin PER1 OS=Zea mays GN=PER1 PE=2 SV=1//2.11976e-154	2277.24	949.58	1.26	4.2E-110
7	GRMZM2G144653	Thioredoxin	GO:0016209//GO:0016491 antioxidant activity//oxidoreductase activity	sp|A2YIW7|TRXH_ORYSI Thioredoxin H-type OS=Oryza sativa subsp. indica GN=TRXH PE=1 SV=1//5.35658e-14	227.67	471.57	-1.05	1.04E-12

7	GRMZM2G151195	#N/A	GO:0050661//GO:0004930//GO:0008883 NADP binding//G-protein coupled receptor activity//glutamyl-tRNA reductase activity	-//-	81.92	40.41	1.02	0.001004

Table 7. Differentially expressed genes associated with plant defence and pathogenesis in maize kernels following inoculation with *Fusarium verticillioides*.

Day(s)	Gene ID	Sequence description	Molecular function and description	Putative annotation (BLAST SwissProt)	Inoculated	Control	Fold change	q value
1	GRMZM2G039383	Nonspecific lipid-transfer protein	GO:0004564//GO:0004575 beta-fructofuranosidase activity//sucrose alpha-glucosidase activity	sp|Q43681|NLTP_VIGUN Probable non-specific lipid-transfer protein AKCS9 OS=Vigna unguiculata PE=2 SV=1//2.11337e-07	26.49	102.92	-1.96	1.9657E-09
1	GRMZM2G083725	Lipid transfer protein	#N/A	-//-	64.1	234.72	-1.87	2.0677E-20
1	GRMZM2G137329	Nonspecific lipid-transfer protein	GO:0004867 serine-type endopeptidase inhibitor activity	sp|A2XBN5|NLTPX_ORYSI Non-specific lipid-transfer protein 2 OS=Oryza sativa subsp. indica GN=LTP-2 PE=2 SV=2//5.20875e-27	53.64	172.55	-1.69	8.516E-13
1	GRMZM2G081464	Probable non-specific lipid-transfer protein 2	#N/A	sp|P83506|NLTP2_MAIZE Probable non-specific lipid-transfer protein 2 OS=Zea mays PE=1 SV=1//7.10853e-39	178.5	552.58	-1.63	1.1946E-39
1	GRMZM2G108153	Uncharacterised protein	GO:0004601//GO:0020037 peroxidase activity//heme binding	sp|P84516|PER1_SORBI Cationic peroxidase SPC4 OS=Sorghum bicolor GN=SORBIDRAFT_03g046810 PE=1 SV=2//2.36469e-73	43.62	134.13	-1.62	2.4447E-09
1	GRMZM2G164440	PVR3-like protein	GO:00515372 iron, 2 sulfur cluster binding	sp|Q8W453|DIR1_ARATH Putative lipid-transfer protein DIR1 OS=Arabidopsis thaliana GN=DIR1 PE=1 SV=1//4.21258e-13	29.6	83.83	-1.5	0.000034871
1	GRMZM2G046459	Uncharacterised protein	GO:0004129//GO:0004553//GO:0003935//GO:0009055 cytochrome-c oxidase activity//hydrolase activity, hydrolyzing O-glycosyl compounds//GTP cyclohydrolase II	sp|Q8VYE5|E1312_ARATH Glucan endo-1,3-beta-glucosidase 12 OS=Arabidopsis thaliana GN=At4g29360 PE=1 SV=1//8.30174e-101	30.94	80.57	-1.38	0.00021021

			activity//electron carrier activity					
1	AC204711.3_FG002	Uncharacterised protein	GO:0004553//GO:0008318//GO:0016798 hydrolase activity, hydrolyzing O-glycosyl compounds//protein prenyltransferase activity//hydrolase activity, acting on glycosyl bonds	sp|O94163|XYNF1_ASPOR Endo-1,4-beta-xylanase F1 OS=Aspergillus oryzae (strain ATCC 42149 / RIB 40) GN=xynF1 PE=1 SV=1//4.57637e-26	59.65	151.13	-1.34	8.7105E-08
1	GRMZM2G101958	Non-specific lipid-transfer protein	#N/A	sp|P19656|NLTP_MAIZE Non-specific lipid-transfer protein OS=Zea mays PE=1 SV=1//1.35417e-40	1919.43	4814.63	-1.33	6.8997E-254
1	GRMZM2G099295	Polygalacturonase inhibitor 1	GO:0005515 protein binding	sp|Q8GT95|PGIP1_ORYSJ Polygalacturonase inhibitor 1 OS=Oryza sativa subsp. japonica GN=FOR1 PE=2 SV=1//4.63234e-176	46.29	115.27	-1.32	9.1068E-06
1	GRMZM2G114552	Uncharacterised protein	GO:0004867 serine-type endopeptidase inhibitor activity	sp|P07679|IBB1_COILA Bowman-Birk type trypsin inhibitor TI1 OS=Coix lachryma-jobi PE=1 SV=1//6.30656e-15	648.57	1568.32	-1.27	1.0206E-76
1	GRMZM2G126397	Nonspecific lipid-transfer protein%3B Putative lipid-transfer protein 3%3B Uncharacterised protein	#N/A	sp|Q9LZV9|NLTPA_ARATH Non-specific lipid-transfer protein 10 OS=Arabidopsis thaliana GN=LTP10 PE=3 SV=1//4.81584e-21	39.17	91.75	-1.23	0.0003853
1	GRMZM2G129777	Putative uncharacterised protein	GO:0003700 sequence-specific DNA binding transcription factor activity	sp|Q3E703|ERF88_ARATH Ethylene-responsive transcription factor ERF088 OS=Arabidopsis thaliana GN=ERF088 PE=2 SV=1//1.23115e-08	186.51	436.85	-1.23	1.0722E-19

1	GRMZM2G059706	Disease resistance response protein 206%3B Uncharacterised protein	#N/A	sp|P13240|DR206_PEA Disease resistance response protein 206 OS=Pisum sativum GN=PI206 PE=2 SV=2//2.39888e-10	32.5	72.65	-1.16	0.0044979
1	GRMZM2G107872	Leucine-rich repeat resistance protein%3B Uncharacterised protein	GO:0005515 protein binding	sp|C0LGQ5|GSO1_ARATH LRR receptor-like serine/threonine-protein kinase GSO1 OS=Arabidopsis thaliana GN=GSO1 PE=2 SV=1//1.53999e-10	44.51	98.5	-1.15	0.00057651
1	GRMZM2G361475	Uncharacterised protein	GO:0001104//GO:0020037//GO:0004601 RNA polymerase II transcription cofactor activity//heme binding//peroxidase activity	sp|P84516|PER1_SORBI Cationic peroxidase SPC4 OS=Sorghum bicolor GN=SORBIDRAFT_03g046810 PE=1 SV=2//3.94777e-147	760.52	1619.31	-1.09	1.1521E-60
1	GRMZM2G368861	Defensin-like protein 2	GO:0019870//GO:0016903 potassium channel inhibitor activity//oxidoreductase activity, acting on the aldehyde or oxo group of donors	sp|P81009|DEF2_MAIZE Defensin-like protein 2 OS=Zea mays PE=1 SV=1//3.79721e-24	4600.28	9809.49	-1.09	0
1	GRMZM2G143791	GPI-anchored protein%3B Uncharacterised protein	GO:0019904//GO:0005213//GO:0008134 protein domain specific binding//structural constituent of chorion//transcription factor binding	-//-	59.65	125.28	-1.07	0.00018626
1	GRMZM2G137535	Lichenase-2%3B Putative O-Glycosyl hydrolase superfamily protein	GO:0004553 hydrolase activity, hydrolyzing O-glycosyl compounds	sp|P12257|GUB2_HORVU Lichenase-2 (Fragment) OS=Hordeum vulgare PE=1 SV=1//2.9089e-173	62.1	129	-1.05	0.00018093
1	GRMZM2G098298	Cysteine proteinase 1	GO:0016625//GO:0004197//GO:0016491//GO:0008234//GO:0051536//GO:0009055 oxidoreductase activity, acting on the aldehyde	sp|Q10716|CYSP1_MAIZE Cysteine proteinase 1 OS=Zea mays GN=CCP1 PE=2 SV=1//0	441.13	898.84	-1.03	5.5397E-30

			or oxo group of donors, iron-sulfur protein as acceptor//cysteine-type endopeptidase activity//oxidoreductase activity//cysteine-type peptidase activity//iron-sulfur cluster binding// electron carrier activity					
1	GRMZM2G075315	Bowman-Birk type trypsin inhibitor%3B Putative Bowman-Birk serine protease inhibitor%3B Uncharacterised protein	GO:0004867 serine-type endopeptidase inhibitor activity	sp|P81713|IBB3_WHEAT Bowman-Birk type trypsin inhibitor OS=Triticum aestivum PE=1 SV=1//3.30714e-10	271.53	90.12	1.59	1.1013E-21
1	GRMZM2G117989	Win1%3B Win1 isoform 1%3B Win1 isoform 2%3B Win1 isoform 3	GO:0005509 calcium ion binding	sp|P43082|HEVL_ARATH Hevein-like protein OS=Arabidopsis thaliana GN=HEL PE=1 SV=1//1.06098e-13	36.72	10.71	1.78	0.0018346
1	GRMZM2G092474	Putative uncharacterised protein	GO:0008233 peptidase activity	sp|P81295|PRR3_JUNAS Pathogenesis-related protein OS=Juniperus ashei PE=1 SV=1//2.52933e-78	592.7	170.22	1.8	1.1427E-56
1	GRMZM2G046532	Flower-specific gamma-thionin%3B Uncharacterised protein	GO:0008200 ion channel inhibitor activity	sp|Q40901|DEF_PETIN Defensin-like protein OS=Petunia integrifolia PE=2 SV=1//6.58806e-18	190.3	51.46	1.89	4.9887E-19
1	GRMZM2G465226	Uncharacterised protein	#N/A	sp|Q05968|PR1_HORVU Pathogenesis-related protein 1 OS=Hordeum vulgare PE=2 SV=1//8.57399e-50	44.51	9.78	2.19	0.000027699
1	GRMZM2G039639	Protein P21	GO:0008233 peptidase activity	sp|P33679|ZEAM_MAIZE Zeamatin OS=Zea mays GN=Zlp PE=1 SV=2//6.10646e-96	57.2	9.08	2.66	2.0147E-08
1	GRMZM2G110289	Uncharacterised protein	GO:0005515 protein binding	sp|Q7RTR2|NLRC3_HUMAN Protein NLRC3 OS=Homo sapiens GN=NLRC3 PE=2 SV=2//5.38345e-25	30.49	3.96	2.95	0.000055053

2	GRMZM2G402631	Uncharacterised protein	GO:0008233 peptidase activity	sp|P31110|TLP_ORYSJ Thaumatin-like protein OS=Oryza sativa subsp. japonica GN=Os12g0628600 PE=1 SV=1//1.142e-76	10.77	251.14	-4.54	5.8046E-58
2	GRMZM2G039639	Protein P21	GO:0008233 peptidase activity	sp|P33679|ZEAM_MAIZE Zeamatin OS=Zea mays GN=Zlp PE=1 SV=2//6.10646e-96	4.31	23.37	-2.44	0.0047116
2	GRMZM2G075315	Bowman-Birk type trypsin inhibitor%3B Putative Bowman-Birk serine protease inhibitor%3B Uncharacterised protein	GO:0004867 serine-type endopeptidase inhibitor activity	sp|P81713|IBB3_WHEAT Bowman-Birk type trypsin inhibitor OS=Triticum aestivum PE=1 SV=1//3.30714e-10	24.88	102.2	-2.04	1.6409E-11
2	GRMZM2G453805	Uncharacterised protein	GO:0004553//GO:0005515//GO:0004357 hydrolase activity, hydrolyzing O-glycosyl compounds//protein binding//glutamate-cysteine ligase activity	sp|P29024|CHIA_PHAAN Acidic endochitinase OS=Phaseolus angularis PE=2 SV=1//1.5657e-115	24.88	80.05	-1.69	5.6803E-07
2	GRMZM2G053206	Uncharacterised protein	#N/A	sp|P13917|7SB1_SOYBN Basic 7S globulin OS=Glycine max GN=BG PE=1 SV=2//1.12155e-08	103.82	288.81	-1.48	3.4565E-22
2	GRMZM2G092474	Putative uncharacterised protein	GO:0008233 peptidase activity	sp|P81295|PRR3_JUNAS Pathogenesis-related protein OS=Juniperus ashei PE=1 SV=1//2.52933e-78	76	178.42	-1.23	2.2699E-10
2	GRMZM2G012160	Cystatin2%3B Cysteine proteinase inhibitor%3B Putative cystatin	GO:0004869 cysteine-type endopeptidase inhibitor activity	sp|P31726|CYT1_MAIZE Cystatin-1 OS=Zea mays GN=RAMDAZC7 PE=2 SV=1//1.24959e-15	90.7	193.94	-1.1	1.5114E-09
2	GRMZM2G083725	Lipid transfer protein	#N/A	-//-	175.12	85.81	1.03	0.000090151
2	GRMZM2G099745	Nitrate-induced NOI protein%3B Uncharacterised protein	#N/A	sp|Q8GYN5|RIN4_ARATH RPM1-interacting protein 4 OS=Arabidopsis thaliana GN=RIN4 PE=1 SV=1//1.72479e-07	222.92	103.77	1.1	5.0926E-07

2	GRMZM2G401328	Cystatin%3B Putative cystatin%3B Uncharacterised protein	GO:0004869 cysteine-type endopeptidase inhibitor activity	sp|Q10J94|CYT8_ORYSJ Cysteine proteinase inhibitor 8 OS=Oryza sativa subsp. japonica GN=Os03g0429000 PE=2 SV=1//3.52954e-37	322.24	141.09	1.19	5.5875E-12
2	GRMZM2G137329	Nonspecific lipid-transfer protein	GO:0004867 serine-type endopeptidase inhibitor activity	sp|A2XBN5|NLTPX_ORYSI Non-specific lipid-transfer protein 2 OS=Oryza sativa subsp. indica GN=LTP-2 PE=2 SV=2//5.20875e-27	153.58	66.27	1.21	0.000010761
2	GRMZM2G081464	Probable non-specific lipid-transfer protein 2	#N/A	sp|P83506|NLTP2_MAIZE Probable non-specific lipid-transfer protein 2 OS=Zea mays PE=1 SV=1//7.10853e-39	613.72	261.96	1.23	8.804E-25
3	GRMZM2G083725	Lipid transfer protein	#N/A	-//-	139.64	67.42	1.05	0.0001189
3	GRMZM2G148925	Cystatin%3B Uncharacterised protein	GO:0004869 cysteine-type endopeptidase inhibitor activity	sp|Q10J94|CYT8_ORYSJ Cysteine proteinase inhibitor 8 OS=Oryza sativa subsp. japonica GN=Os03g0429000 PE=2 SV=1//3.4387e-34	890.8	428.95	1.05	3.8429E-31
3	GRMZM2G368861	Defensin-like protein 2	GO:0019870//GO:0016903 potassium channel inhibitor activity//oxidoreductase activity, acting on the aldehyde or oxo group of donors	sp|P81009|DEF2_MAIZE Defensin-like protein 2 OS=Zea mays PE=1 SV=1//3.79721e-24	8230.54	3886.47	1.08	0
3	GRMZM2G041039	Uncharacterised protein	GO:0005198 structural molecule activity	-//-	93.89	42.14	1.16	0.0010555
3	GRMZM2G114552	Uncharacterised protein	GO:0004867 serine-type endopeptidase inhibitor activity	sp|P07679|IBB1_COILA Bowman-Birk type trypsin inhibitor TI1 OS=Coix lachryma-jobi PE=1 SV=1//6.30656e-15	835.66	365.24	1.19	2.6521E-36
3	GRMZM2G137329	Nonspecific lipid-transfer protein	GO:0004867 serine-type endopeptidase inhibitor activity	sp|A2XBN5|NLTPX_ORYSI Non-specific lipid-transfer protein 2 OS=Oryza sativa subsp. indica GN=LTP-2 PE=2 SV=2//5.20875e-27	155.82	68.09	1.19	1.7441E-06

3	GRMZM2G011523	Bowman-Birk type wound-induced proteinase inhibitor WIP1%3B Uncharacterised protein	#N/A	-//-	339.42	143.77	1.24	2.0994E-15
3	GRMZM2G361475	Uncharacterised protein	GO:0001104//GO:0020037//GO:0004601 RNA polymerase II transcription cofactor activity//heme binding//peroxidase activity	sp|P84516|PER1_SORBI Cationic peroxidase SPC4 OS=Sorghum bicolor GN=SORBIDRAFT_03g046810 PE=1 SV=2//3.94777e-147	2064.28	876.77	1.24	3.0524E-96
3	GRMZM2G101958	Non-specific lipid-transfer protein	#N/A	sp|P19656|NLTP_MAIZE Non-specific lipid-transfer protein OS=Zea mays PE=1 SV=1//1.35417e-40	3353.63	1357.63	1.3	7.6883E-172
3	GRMZM2G164440	PVR3-like protein	GO:00515372 iron, 2 sulfur cluster binding	sp|Q8W453|DIR1_ARATH Putative lipid-transfer protein DIR1 OS=Arabidopsis thaliana GN=DIR1 PE=1 SV=1//4.21258e-13	70.12	27.98	1.33	0.001749
3	GRMZM2G010868	Non-specific lipid-transfer protein	#N/A	sp|Q43194|NLTP2_SORBI Non-specific lipid-transfer protein 2 OS=Sorghum bicolor GN=LTP2 PE=3 SV=1//5.34955e-42	262.5	103.99	1.34	1.9608E-13
3	GRMZM2G177792	Peroxidase 1	GO:0020037//GO:0004601//GO:0042302 heme binding//peroxidase activity//structural constituent of cuticle	sp|A7QEU4|PER5_VITVI Peroxidase 5 OS=Vitis vinifera GN=GSVIVT00037159001 PE=1 SV=2//1.59091e-105	165.81	65.23	1.35	1.6845E-08
3	GRMZM2G374971	Zeamatin	GO:0008233 peptidase activity	sp|P33679|ZEAM_MAIZE Zeamatin OS=Zea mays GN=Zlp PE=1 SV=2//1.48542e-152	467.07	182.2	1.36	5.9528E-25
3	GRMZM2G137535	Lichenase-2%3B Putative O-Glycosyl hydrolase superfamily protein	GO:0004553 hydrolase activity, hydrolyzing O-glycosyl compounds	sp|P12257|GUB2_HORVU Lichenase-2 (Fragment) OS=Hordeum vulgare PE=1 SV=1//2.9089e-173	164.02	61.52	1.41	4.1217E-09
3	GRMZM2G081464	Probable non-specific lipid-transfer protein 2	#N/A	sp|P83506|NLTP2_MAIZE Probable non-specific lipid-transfer protein 2 OS=Zea mays PE=1 SV=1//7.10853e-	506.03	187.42	1.43	1.5432E-29

3	GRMZM2G092474	Putative uncharacterised protein	GO:0008233 peptidase activity	sp|P81295|PRR3_JUNAS Pathogenesis-related protein OS=Juniperus ashei PE=1 SV=1//2.52933e-78	236.93	73.32	1.69	1.1108E-17
3	GRMZM2G447795	Xylanase inhibitor protein 1	GO:0004553 hydrolase activity, hydrolyzing O-glycosyl compounds	sp|Q7GCM7|XIP1_ORYSJ Xylanase inhibitor protein 1 OS=Oryza sativa subsp. japonica GN=RIXI PE=1 SV=1//9.51582e-108	93.49	27.14	1.78	2.3106E-07
3	GRMZM2G125032	Beta-1%2C3-glucanase	GO:0004553 hydrolase activity, hydrolyzing O-glycosyl compounds	sp|P15737|E13B_HORVU Glucan endo-1,3-beta-glucosidase GII OS=Hordeum vulgare PE=1 SV=1//1.31787e-155	252.52	59.5	2.09	1.5881E-25
3	GRMZM2G051943	Endochitinase A	GO:0004568//GO:0008061 chitinase activity//chitin binding	sp|P29022|CHIA_MAIZE Endochitinase A OS=Zea mays PE=1 SV=1//5.71989e-171	1547.26	321.25	2.27	2.0661E-178
7	GRMZM2G164440	PVR3-like protein	GO:00515372 iron, 2 sulfur cluster binding	sp|Q8W453|DIR1_ARATH Putative lipid-transfer protein DIR1 OS=Arabidopsis thaliana GN=DIR1 PE=1 SV=1//4.21258e-13	30.15	99.12	-1.72	2.8627E-06
7	GRMZM2G401328	Cystatin%3B Putative cystatin%3B Uncharacterised protein	GO:0004869 cysteine-type endopeptidase inhibitor activity	sp|Q10J94|CYT8_ORYSJ Cysteine proteinase inhibitor 8 OS=Oryza sativa subsp. japonica GN=Os03g0429000 PE=2 SV=1//3.52954e-37	85.87	232.73	-1.44	3.0276E-11
7	GRMZM2G114552	Uncharacterised protein	GO:0004867 serine-type endopeptidase inhibitor activity	sp|P07679|IBB1_COILA Bowman-Birk type trypsin inhibitor TI1 OS=Coix lachryma-jobi PE=1 SV=1//6.30656e-15	296.07	655.88	-1.15	1.6873E-21
7	GRMZM2G137839	APx1-Cytosolic Ascorbate Peroxidase%3B Ascorbate peroxidase	GO:0020037//GO:0004601 heme binding//peroxidase activity	sp|Q10N21|APX1_ORYSJ L-ascorbate peroxidase 1, cytosolic OS=Oryza sativa subsp. japonica GN=APX1 PE=1 SV=1//1.82698e-163	166.33	364.25	-1.13	2.7289E-11

7	GRMZM2G081464	Probable non-specific lipid-transfer protein 2	#N/A	sp|P83506|NLTP2_MAIZE Probable non-specific lipid-transfer protein 2 OS=Zea mays PE=1 SV=1//7.10853e-39	292.75	631.1	-1.11	2.8773E-19
7	GRMZM2G039383	Nonspecific lipid-transfer protein	GO:0004564//GO:0004575 beta-fructofuranosidase activity//sucrose alpha-glucosidase activity	sp|Q43681|NLTP_VIGUN Probable non-specific lipid-transfer protein AKCS9 OS=Vigna unguiculata PE=2 SV=1//2.11337e-07	97.72	205.48	-1.07	0.00001386
7	GRMZM2G137329	Nonspecific lipid-transfer protein	GO:0004867 serine-type endopeptidase inhibitor activity	sp|A2XBN5|NLTPX_ORYSI Non-specific lipid-transfer protein 2 OS=Oryza sativa subsp. indica GN=LTP-2 PE=2 SV=2//5.20875e-27	87.95	177.46	-1.01	0.00030369

Table 8. Differentially expressed genes associated with other defence-related events in maize kernels following inoculation with *Fusarium verticillioides*.

Day(s)	Gene ID	Sequence description	Molecular function and description	Putative annotation (BLAST SwissProt)	Inoculated	Control	Fold change	q value
1	AC209206.3_FG014	Putative polyphenol oxidase family protein	GO:0004097//GO:0030550//GO:0016491 catechol oxidase activity//acetylcholine receptor inhibitor activity//oxidoreductase activity	sp|Q9FRX6|AS1_ANTMA Aureusidin synthase OS=Antirrhinum majus GN=AS1 PE=1 SV=1//1.60558e-22	92.59	92.59	-1.61	8.5526E-20
1	GRMZM2G445169	Uncharacterised protein	GO:0016788 hydrolase activity, acting on ester bonds	sp|Q852A1|EXPA7_ORYSJ Expansin-A7 OS=Oryza sativa subsp. japonica GN=EXPA7 PE=2 SV=1//6.12587e-91	37.17	37.17	-1.44	7.3076E-06
1	GRMZM2G339122	Alpha-expansin 1%3B Alpha-expansin 10%3B Uncharacterised protein	#N/A	sp|Q0DHB7|EXPA4_ORYSJ Expansin-A4 OS=Oryza sativa subsp. japonica GN=EXPA4 PE=2 SV=1//4.06371e-63	24.48	24.48	-1.31	0.0038063
1	GRMZM2G176595	Beta-expansin 1a%3B Beta-expansin 6%3B Uncharacterised protein	#N/A	sp|Q336T5|EXPB3_ORYSJ Expansin-B3 OS=Oryza sativa subsp. japonica GN=EXPB3 PE=2 SV=2//1.23961e-15	64.1	64.1	-1.07	0.00010366
1	GRMZM2G148485	Uncharacterised protein	#N/A	sp|Q7XT40|EXB15_ORYSJ Expansin-B15 OS=Oryza sativa subsp. japonica GN=EXPB15 PE=3 SV=2//6.04589e-109	76.56	76.56	-1.05	2.2261E-05
1	GRMZM2G073054	Starch branching enzyme IIa	GO:0004553//GO:0003824//GO:0043169 hydrolase activity, hydrolyzing O-glycosyl compounds//catalytic activity//cation binding	sp|Q08047|GLGB_MAIZE 1,4-alpha-glucan-branching enzyme 2, chloroplastic/amyloplastic OS=Zea mays GN=SBE1 PE=1 SV=1//0	146.01	146.01	1.1	1.0631E-06

1	GRMZM5G840560	#N/A	GO:0005524//GO:0003724//GO:0003723//GO:0003843 ATP binding//RNA helicase activity//RNA binding//1,3-beta-D-glucan synthase activity	sp|Q9LXT9|CALS3_ARATH Callose synthase 3 OS=Arabidopsis thaliana GN=CALS3 PE=2 SV=3//0	81.46	81.46	1.2	0.0002426
1	GRMZM2G453794	#N/A	GO:0003843 1,3-beta-D-glucan synthase activity	sp|Q9SFU6|CALS9_ARATH Callose synthase 9 OS=Arabidopsis thaliana GN=CALS9 PE=2 SV=2//0	124.86	124.86	1.21	1.2789E-06
1	GRMZM2G067315	#N/A	GO:0016814//GO:0008270 hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amidines//zinc ion binding	-//-	510.35	510.35	1.32	1.9653E-31
1	GRMZM2G140917	Uncharacterised protein	----	sp|Q86TW2|ADCK1_HUMAN Uncharacterised aarF domain-containing protein kinase 1 OS=Homo sapiens GN=ADCK1 PE=1 SV=2//7.16436e-46	52.97	52.97	1.32	0.00253
1	GRMZM2G430680	Putative glycosyl transferase family protein	GO:0003843 1,3-beta-D-glucan synthase activity	sp|Q9LXT9|CALS3_ARATH Callose synthase 3 OS=Arabidopsis thaliana GN=CALS3 PE=2 SV=3//0	52.08	52.08	1.5	0.00074116
1	GRMZM2G326643	Uncharacterised protein	GO:0003843 1,3-beta-D-glucan synthase activity	sp|Q9SJM0|CALSA_ARATH Callose synthase 10 OS=Arabidopsis thaliana GN=CALS10 PE=2 SV=5//0	126.42	126.42	1.52	2.1821E-09
1	GRMZM2G032628	1%2C4-alpha-glucan-branching enzyme 2%2C chloroplastic/amyloplastic	GO:0004553//GO:0003824//GO:0043169 hydrolase activity, hydrolyzing O-glycosyl compounds//catalytic activity//cation binding	sp|Q08047|GLGB_MAIZE 1,4-alpha-glucan-branching enzyme 2, chloroplastic/amyloplastic OS=Zea mays GN=SBE1 PE=1 SV=1//7.75312e-11	1108.17	1108.17	1.58	5.4575E-89
1	GRMZM2G355523	#N/A	GO:0005524//GO:0016887 ATP binding//ATPase activity	sp|Q9STT5|AB7A_ARATH ABC transporter A family member 7 OS=Arabidopsis thaliana GN=ABCA7 PE=1 SV=2//0	25.15	25.15	2.23	0.0035539

1	GRMZM2G180951	Uncharacterised protein	GO:0003843 1,3-beta-D-glucan synthase activity	sp|Q9AUE0|CALS1_ARATH Callose synthase 1 OS=Arabidopsis thaliana GN=CALS1 PE=1 SV=2//1.23232e-12	40.51	40.51	2.36	3.0473E-05
1	GRMZM2G084802	#N/A	GO:0003843 1,3-beta-D-glucan synthase activity	sp|Q9LYS6|CALS6_ARATH Putative callose synthase 6 OS=Arabidopsis thaliana GN=CALS6 PE=3 SV=2//0	17.14	17.14	5.2	0.00055664
2	GRMZM2G067315	#N/A	GO:0016814//GO:00082 70 hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amidines//zinc ion binding	-//-	108.33	108.33	-1.45	3.1556E-22
2	GRMZM2G326643	Uncharacterised protein	GO:0003843 1,3-beta-D-glucan synthase activity	sp|Q9SJM0|CALSA_ARATH Callose synthase 10 OS=Arabidopsis thaliana GN=CALS10 PE=2 SV=5//0	37.41	37.41	-1.23	6.4498E-05
2	GRMZM2G445169	Uncharacterised protein	GO:0016788 hydrolase activity, acting on ester bonds	sp|Q852A1|EXPA7_ORYSJ Expansin-A7 OS=Oryza sativa subsp. japonica GN=EXPA7 PE=2 SV=1//6.12587e-91	91.48	91.48	1.17	0.0040225
3	GRMZM2G032628	1%2C4-alpha-glucan-branching enzyme 2%2C chloroplastic/amyloplastic	GO:0004553//GO:00038 24//GO:0043169 hydrolase activity, hydrolyzing O-glycosyl compounds//catalytic activity//cation binding	sp|Q08047|GLGB_MAIZE 1,4-alpha-glucan-branching enzyme 2, chloroplastic/amyloplastic OS=Zea mays GN=SBE1 PE=1 SV=1//7.75312e-11	299.66	299.66	-1.69	1.2939E-85
3	GRMZM2G326643	Uncharacterised protein	GO:0003843 1,3-beta-D-glucan synthase activity	sp|Q9SJM0|CALSA_ARATH Callose synthase 10 OS=Arabidopsis thaliana GN=CALS10 PE=2 SV=5//0	47.75	47.75	-1.43	1.3901E-08
3	GRMZM2G088753	1%2C4-alpha-glucan branching enzyme%3B Starch branching enzyme I%3B Uncharacterised protein	GO:0004553//GO:00038 24//GO:0043169 hydrolase activity, hydrolyzing O-glycosyl compounds//catalytic activity//cation binding	sp|Q01401|GLGB_ORYSJ 1,4-alpha-glucan-branching enzyme, chloroplastic/amyloplastic OS=Oryza sativa subsp. japonica GN=SBE1 PE=1 SV=2//2.48311e-11	407.34	407.34	-1.1	8.9609E-41

3	GRMZM2G026956	#N/A	GO:0051920 peroxiredoxin activity	sp|Q7XT40|EXB15_ORYSJ Expansin-B15 OS=Oryza sativa subsp. japonica GN=EXPB15 PE=3 SV=2//1.42234e-107	365.19	365.19	1.22	3.5348E-16
3	GRMZM2G176595	Beta-expansin 1a%3B Beta-expansin 6%3B Uncharacterised protein	#N/A	sp|Q336T5|EXPB3_ORYSJ Expansin-B3 OS=Oryza sativa subsp. japonica GN=EXPB3 PE=2 SV=2//1.23961e-15	140.24	140.24	1.22	4.286E-06
3	GRMZM2G445169	Uncharacterised protein	GO:0016788 hydrolase activity, acting on ester bonds	sp|Q852A1|EXPA7_ORYSJ Expansin-A7 OS=Oryza sativa subsp. japonica GN=EXPA7 PE=2 SV=1//6.12587e-91	92.3	92.3	1.27	0.00029797
3	GRMZM2G148485	Uncharacterised protein	#N/A	sp|Q7XT40|EXB15_ORYSJ Expansin-B15 OS=Oryza sativa subsp. japonica GN=EXPB15 PE=3 SV=2//6.04589e-109	180	180	1.3	9.6608E-09
3	GRMZM2G094990	Beta-expansin 1a%3B Uncharacterised protein	#N/A	sp|Q336T5|EXPB3_ORYSJ Expansin-B3 OS=Oryza sativa subsp. japonica GN=EXPB3 PE=2 SV=2//1.25258e-124	150.63	150.63	1.94	1.4255E-13
7	GRMZM2G339122	Alpha-expansin 1%3B Alpha-expansin 10%3B Uncharacterised protein	#N/A	sp|Q0DHB7|EXPA4_ORYSJ Expansin-A4 OS=Oryza sativa subsp. japonica GN=EXPA4 PE=2 SV=1//4.06371e-63	13.93	13.93	-2.05	9.9368E-05
7	GRMZM2G094990	Beta-expansin 1a%3B Uncharacterised protein	#N/A	sp|Q336T5|EXPB3_ORYSJ Expansin-B3 OS=Oryza sativa subsp. japonica GN=EXPB3 PE=2 SV=2//1.25258e-124	28.48	28.48	-1.95	1.157E-08
7	GRMZM2G176595	Beta-expansin 1a%3B Beta-expansin 6%3B Uncharacterised protein	#N/A	sp|Q336T5|EXPB3_ORYSJ Expansin-B3 OS=Oryza sativa subsp. japonica GN=EXPB3 PE=2 SV=2//1.23961e-15	36.59	36.59	-1.55	8.7554E-06
7	AC209206.3_FG014	Putative polyphenol oxidase family protein	GO:0004097//GO:0030550//GO:0016491 catechol oxidase activity//acetylcholine receptor inhibitor activity//oxidoreductase activity	sp|Q9FRX6|AS1_ANTMA Aureusidin synthase OS=Antirrhinum majus GN=AS1 PE=1 SV=1//1.60558e-22	138.27	138.27	-1.38	2.0239E-16

7	GRMZM2G445169	Uncharacterised protein	GO:0016788 hydrolase activity, acting on ester bonds	sp|Q852A1|EXPA7_ORYSJ Expansin-A7 OS=Oryza sativa subsp. japonica GN=EXPA7 PE=2 SV=1//6.12587e-91	51.56	51.56	-1.22	0.00035789
7	GRMZM2G148485	Uncharacterised protein	#N/A	sp|Q7XT40|EXB15_ORYSJ Expansin-B15 OS=Oryza sativa subsp. japonica GN=EXPB15 PE=3 SV=2//6.04589e-109	109.16	109.16	-1.21	9.5147E-09
7	GRMZM2G026956	#N/A	GO:0051920 peroxiredoxin activity	sp|Q7XT40|EXB15_ORYSJ Expansin-B15 OS=Oryza sativa subsp. japonica GN=EXPB15 PE=3 SV=2//1.42234e-107	253.87	253.87	-1.2	1.4058E-20
7	GRMZM2G140917	Uncharacterised protein	----	sp|Q86TW2|ADCK1_HUMAN Uncharacterised aarF domain-containing protein kinase 1 OS=Homo sapiens GN=ADCK1 PE=1 SV=2//7.16436e-46	81.71	81.71	1.08	0.00050024

Table 9. Differentially expressed genes associated with hormone-based signalling in maize kernels following inoculation with *Fusarium verticillioides*.

Day(s)	Gene ID	Sequence description	Molecular function and description	Putative annotation (BLAST SwissProt)	Inoculated	Control	Fold change	q value
1	GRMZM2G049418	Uncharacterised protein	GO:0051536//GO:0016491// GO:0016706 iron-sulfur cluster binding//oxidoreductase activity//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	sp|Q0JH50|GAOX2_ORYSJ Gibberellin 20 oxidase 2 OS=Oryza sativa subsp. japonica GN=20ox2 PE=1 SV=1//4.43154e-33	4.23	24.45	-2.53	0.003168
1	GRMZM2G085964	Putative AP2/EREBP transcription factor superfamily protein%3B Uncharacterised protein	GO:0003700 sequence-specific DNA binding transcription factor activity	sp|P42736|RAP23_ARATH Ethylene-responsive transcription factor RAP2-3 OS=Arabidopsis thaliana GN=RAP2-3 PE=1 SV=2//3.26147e-24	13.13	60.08	-2.19	2.14E-06
1	GRMZM2G318689	Uncharacterised protein	GO:0005515//GO:0000156 protein binding//two-component response regulator activity	sp|Q0WPQ2|ETR2_ARATH Ethylene receptor 2 OS=Arabidopsis thaliana GN=ETR2 PE=1 SV=2//9.48659e-22	9.35	35.86	-1.94	0.0023855
1	GRMZM2G033359	Uncharacterised protein	#N/A	sp|P0C0M2|GH32_ORYSJ Probable indole-3-acetic acid-amido synthetase GH3.2 OS=Oryza sativa subsp. japonica GN=GH3.2 PE=2 SV=1//5.40639e-11	34.5	122.25	-1.83	3.728E-10
1	GRMZM2G079470	Uncharacterised protein	#N/A	sp|O81316|SCL6_ARATH Scarecrow-like protein 6 OS=Arabidopsis thaliana GN=SCL6 PE=1 SV=1//1.85666e-75	18.47	58.91	-1.67	0.0002426
1	GRMZM2G068202	Gibberellin-regulated protein 2	#N/A	sp|P46687|GASA3_ARATH Gibberellin-regulated protein 3 OS=Arabidopsis thaliana GN=GASA3 PE=2 SV=1//1.09264e-13	15.13	45.41	-1.59	0.0035867

1	GRMZM2G121700	Uncharacterised protein	GO:0016491//GO:0016706// GO:0003677 oxidoreductase activity//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors//DNA binding	sp|O04706|GAO1B_WHEAT Gibberellin 20 oxidase 1-B OS=Triticum aestivum GN=GA20ox1B PE=2 SV=1//8.55706e-18	108.39	323.21	-1.58	8.211E-22
1	GRMZM2G174347	Ethylene-responsive transcription factor 4%3B Putative AP2/EREBP transcription factor superfamily protein	GO:0003700//GO:0046789// GO:0016788 sequence-specific DNA binding transcription factor activity//host cell surface receptor binding//hydrolase activity, acting on ester bonds	sp|Q9LW49|ERF4_NICSY Ethylene-responsive transcription factor 4 OS=Nicotiana sylvestris GN=ERF4 PE=2 SV=1//1.69988e-15	100.82	292.94	-1.54	5.009E-19
1	GRMZM2G053338	Uncharacterised protein	#N/A	sp|Q0D4Z6|GH38_ORYSJ Probable indole-3-acetic acid-amido synthetase GH3.8 OS=Oryza sativa subsp. japonica GN=GH3.8 PE=2 SV=1//4.69941e-56	77.23	220.29	-1.51	7.448E-14
1	GRMZM2G378106	Indole-3-acetic acid amido synthetase	GO:0008200 ion channel inhibitor activity	sp|Q0D4Z6|GH38_ORYSJ Probable indole-3-acetic acid-amido synthetase GH3.8 OS=Oryza sativa subsp. japonica GN=GH3.8 PE=2 SV=1//1.56456e-58	218.56	624.3	-1.51	5.401E-40
1	GRMZM2G079949	Uncharacterised protein	GO:0016787 hydrolase activity	sp|Q9SX78|CXE2_ARATH Probable carboxylesterase 2 OS=Arabidopsis thaliana GN=CXE2 PE=2 SV=1//9.84163e-58	22.26	59.38	-1.42	0.0019029
1	GRMZM2G126732	Uncharacterised protein	#N/A	sp|A2Z1W9|ACCO1_ORYSI 1-aminocyclopropane-1-carboxylate oxidase 1 OS=Oryza sativa subsp. indica GN=ACO1 PE=2 SV=1//1.87944e-09	204.76	546.29	-1.42	2.486E-31
1	GRMZM2G144744	DELLA protein DWARF8	GO:0003677 DNA binding	sp|Q9ST48|DWRF8_MAIZE DELLA protein DWARF8 OS=Zea mays GN=D8 PE=1 SV=1//2.25314e-51	32.27	81.73	-1.34	0.0002929

1	GRMZM2G112238	Jasmonate-induced protein%3B Uncharacterised protein	----	sp|A2WPN7|SALT_ORYSI Salt stress-induced protein OS=Oryza sativa subsp. indica GN=SALT PE=1 SV=2//5.32595e-07	220.12	525.8	-1.26	1.016E-24
1	GRMZM2G172204	Uncharacterised protein	#N/A	sp|P83304|LEC_PARPC Mannose/glucose-specific lectin (Fragment) OS=Parkia platycephala PE=1 SV=1//1.32077e-10	30.27	71.72	-1.24	0.0022338
1	GRMZM2G077008	#N/A	GO:0005515 protein binding	sp|Q0WPQ2|ETR2_ARATH Ethylene receptor 2 OS=Arabidopsis thaliana GN=ETR2 PE=1 SV=2//3.4359e-12	91.7	190.71	-1.06	1.477E-06
1	GRMZM2G050851	Uncharacterised protein	GO:0003700 sequence-specific DNA binding transcription factor activity	sp|O22259|ERF71_ARATH Ethylene-responsive transcription factor ERF071 OS=Arabidopsis thaliana GN=ERF071 PE=2 SV=1//3.84618e-21	101.94	48.9	1.06	0.0001756
1	GRMZM2G137413	Uncharacterised protein	GO:0003677 DNA binding	sp|Q6YVY0|ARFG_ORYSJ Auxin response factor 7 OS=Oryza sativa subsp. japonica GN=ARF7 PE=2 SV=1//0	33.61	8.85	1.93	0.0016672
1	GRMZM2G167741	Uncharacterised protein	GO:0016810 hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	sp|Q9Z0W3|NU160_MOUSE Nuclear pore complex protein Nup160 OS=Mus musculus GN=Nup160 PE=1 SV=2//7.9148e-23	50.97	10.71	2.25	2.986E-06
2	GRMZM2G112238	Jasmonate-induced protein%3B Uncharacterised protein	----	sp|A2WPN7|SALT_ORYSI Salt stress-induced protein OS=Oryza sativa subsp. indica GN=SALT PE=1 SV=2//5.32595e-07	477.77	221.49	1.11	9.359E-16
3	GRMZM2G112238	Jasmonate-induced protein%3B Uncharacterised protein	----	sp|A2WPN7|SALT_ORYSI Salt stress-induced protein OS=Oryza sativa subsp. indica GN=SALT PE=1 SV=2//5.32595e-07	13.98	113.43	-3.02	4.724E-19
3	GRMZM2G050851	Uncharacterised protein	GO:0003700 sequence-specific DNA binding transcription factor activity	sp|O22259|ERF71_ARATH Ethylene-responsive transcription factor ERF071 OS=Arabidopsis thaliana GN=ERF071 PE=2 SV=1//3.84618e-21	59.93	124.89	-1.06	2.692E-05
3	GRMZM2G145974	Ethylene-responsive protein	----	-//-	570.16	282.65	1.01	2.296E-18
3	GRMZM2G121700	Uncharacterised protein	GO:0016491//GO:0016706//GO:0003677 oxidoreductase activity//oxidoreductase activity, acting on paired	sp|O04706|GAO1B_WHEAT Gibberellin 20 oxidase 1-B OS=Triticum aestivum GN=GA20ox1B PE=2 SV=1//8.55706e-18	191.18	86.29	1.15	1.8E-07

			donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors//DNA binding					
7	GRMZM2G475683	SAUR12-auxin-responsive SAUR family member	----	sp|P32295|ARG7_VIGRR Indole-3-acetic acid-induced protein ARG7 OS=Vigna radiata var. radiata GN=ARG7 PE=2 SV=1//4.94836e-10	4.57	32.02	-2.81	0.0005759
7	GRMZM2G053338	Uncharacterised protein	#N/A	sp|Q0D4Z6|GH38_ORYSJ Probable indole-3-acetic acid-amido synthetase GH3.8 OS=Oryza sativa subsp. japonica GN=GH3.8 PE=2 SV=1//4.69941e-56	74.85	221.68	-1.57	1.851E-12
7	GRMZM2G033359	Uncharacterised protein	#N/A	sp|P0C0M2|GH32_ORYSJ Probable indole-3-acetic acid-amido synthetase GH3.2 OS=Oryza sativa subsp. japonica GN=GH3.2 PE=2 SV=1//5.40639e-11	58.42	134.38	-1.2	0.0001684
7	GRMZM2G312110	#N/A	GO:0008270 zinc ion binding	sp|B9G2A8|BIG_ORYSJ Auxin transport protein BIG OS=Oryza sativa subsp. japonica GN=Os09g0247700 PE=2 SV=1//0	97.51	37.74	1.37	1.041E-06

Table 10. Differentially expressed genes associated with secondary metabolism in maize kernels following inoculation with *Fusarium verticillioides*.

Day(s)	Gene ID	Sequence description	Molecular function and description	Putative annotation (BLAST SwissProt)	Inoculated	Control	Fold change	q value
1	GRMZM2G081582	Phenylalanine ammonia-lyase	GO:0016841 ammonia-lyase activity	sp|A2X7F7|PAL2_ORYSI Phenylalanine ammonia-lyase OS=Oryza sativa subsp. indica GN=ZB8 PE=3 SV=1//8.22882e-44	31.83	113.64	-1.84	1.6382E-09
1	GRMZM2G066142	Agmatine coumaroyltransferase%3B Uncharacterised protein	GO:0016747 transferase activity, transferring acyl groups other than amino-acyl groups	sp|Q94CD1|HHT1_ARATH Omega-hydroxypalmitate O-feruloyl transferase OS=Arabidopsis thaliana GN=HHT1 PE=1 SV=1//2.78908e-15	28.49	85.69	-1.59	8.8558E-06
1	GRMZM2G066049	Agmatine coumaroyltransferase	GO:0003700//GO:0016747 sequence-specific DNA binding transcription factor activity//transferase activity, transferring acyl groups other than amino-acyl groups	sp|Q94CD1|HHT1_ARATH Omega-hydroxypalmitate O-feruloyl transferase OS=Arabidopsis thaliana GN=HHT1 PE=1 SV=1//5.35016e-18	81.91	234.72	-1.52	7.1115E-15
1	GRMZM2G013530	Agmatine coumaroyltransferase	GO:0016747//GO:0016787 transferase activity, transferring acyl groups other than amino-acyl groups//hydrolase activity	sp|A9ZPJ7|AGCT2_HORVU Agmatine coumaroyltransferase-2 OS=Hordeum vulgare GN=ACT-2 PE=1 SV=1//8.89236e-140	69	185.59	-1.43	1.257E-10
1	GRMZM2G074604	Phenylalanine ammonia-lyase	GO:0016841 ammonia-lyase activity	sp|Q8VXG7|PALY_MAIZE Phenylalanine/tyrosine ammonia-lyase OS=Zea mays GN=PAL1 PE=1 SV=1//8.33268e-75	350.77	920.5	-1.39	8.487E-52
1	GRMZM2G029048	Putative uncharacterised protein	GO:0016841 ammonia-lyase activity	sp|A2X7F7|PAL2_ORYSI Phenylalanine ammonia-lyase OS=Oryza sativa subsp. indica GN=ZB8 PE=3 SV=1//8.9029e-43	140.89	340.21	-1.27	3.1255E-16
1	GRMZM2G127087	(S)-beta-macrocarpene synthase	GO:0000287//GO:0003712// GO:0010333//GO:0004402// GO:0008270//GO:0016829	sp|Q1EG72|TPS11_MAIZE (S)-beta-macrocarpene synthase OS=Zea mays GN=TPS11	320.5	119.46	1.42	6.7405E-22

			magnesium ion binding// transcription cofactor activity //terpene synthase activity // histone acetyltransferase activity// zinc ion binding //lyase activity	PE=1 SV=1//6.70769e-20				
1	GRMZM2G175076	Chalcone--flavonone isomerase%3B; Un-characterised protein	GO:0016872 intramolecular lyase activity	-//-	32.5	8.85	1.88	0.0026458
1	AC214360.3_FG001	Uncharacterised protein	GO:0010333//GO:0003824// GO:0000287//GO:0016829 terpene synthase activity//catalytic activity//magnesium ion binding//lyase activity	sp|Q0JA82|KS1_ORYSJ Ent-kaur-16-ene synthase, chloroplastic OS=Oryza sativa subsp. japonica GN=KS1 PE=1 SV=1//3.36563e-07	52.75	11.18	2.24	1.9961E-06
2	GRMZM2G127087	(S)-beta-macrocarpene synthase	GO:0000287//GO:0003712// GO:0010333//GO:0004402// GO:0008270//GO:0016829 magnesium ion binding//transcription cofactor activity//terpene synthase activity//histone acetyltransferase activity//zinc ion binding//lyase activity	sp|Q1EG72|TPS11_MAIZE (S)-beta-macrocarpene synthase OS=Zea mays GN=TPS11 PE=1 SV=1//6.70769e-20	9.6	104.12	-3.44	4.57E-20
2	AC214360.3_FG001	Uncharacterised protein	GO:0010333//GO:0003824// GO:0000287//GO:0016829 terpene synthase activity//catalytic activity//magnesium ion binding//lyase activity	sp|Q0JA82|KS1_ORYSJ Ent-kaur-16-ene synthase, chloroplastic OS=Oryza sativa subsp. japonica GN=KS1 PE=1 SV=1//3.36563e-07	3.72	27.56	-2.89	0.00026281
3	GRMZM5G814904	Uncharacterised protein	GO:0008168//GO:0046983// GO:0008171 methyltransferase activity//protein dimerization activity//O-methyltransferase activity	sp|Q06509|COMT1_MAIZE Caffeic acid 3-O-methyltransferase OS=Zea mays PE=3 SV=1//0	372.38	184.05	1.02	6.0917E-12
3	GRMZM2G081582	Phenylalanine ammonia-lyase	GO:0016841 ammonia-lyase activity	sp|A2X7F7|PAL2_ORYSI Phenylalanine ammonia-lyase OS=Oryza sativa subsp. indica GN=ZB8 PE=3 SV=1//8.22882e-44	103.08	49.05	1.07	0.0014183
3	GRMZM2G174347	Ethylene-responsive	GO:0003700//GO:0046789//	sp|Q9LW49|ERF4_NICSY	225.95	99.27	1.19	2.1232E-09

		transcription factor 4%3B Putative AP2/EREBP transcription factor superfamily protein	GO:0016788 sequence-specific DNA binding transcription factor activity//host cell surface receptor binding//hydrolase activity, acting on ester bonds	Ethylene-responsive transcription factor 4 OS=Nicotiana sylvestris GN=ERF4 PE=2 SV=1//1.69988e-15				
7	GRMZM2G013530	Agmatine coumaroyltransferase	GO:0016747//GO:0016787 transferase activity, transferring acyl groups other than amino-acyl groups//hydrolase activity	sp|A9ZPJ7|AGCT2_HORVU Agmatine coumaroyltransferase-2 OS=Hordeum vulgare GN=ACT-2 PE=1 SV=1//8.89236e-140	63.41	155.54	-1.29	4.6977E-06
7	GRMZM2G121700	Uncharacterised protein	GO:0016491//GO:0016706//GO:0003677 oxidoreductase activity//oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors//DNA binding	sp|O04706|GAO1B_WHEAT Gibberellin 20 oxidase 1-B OS=Triticum aestivum GN=GA20ox1B PE=2 SV=1//8.55706e-18	122.05	299.45	-1.29	4.9368E-12
7	GRMZM5G814904	Uncharacterised protein	GO:0008168//GO:0046983//GO:0008171 methyltransferase activity//protein dimerization activity//O-methyltransferase activity	sp|Q06509|COMT1_MAIZE Caffeic acid 3-O-methyltransferase OS=Zea mays PE=3 SV=1//0	163.63	354.15	-1.11	1.2493E-10

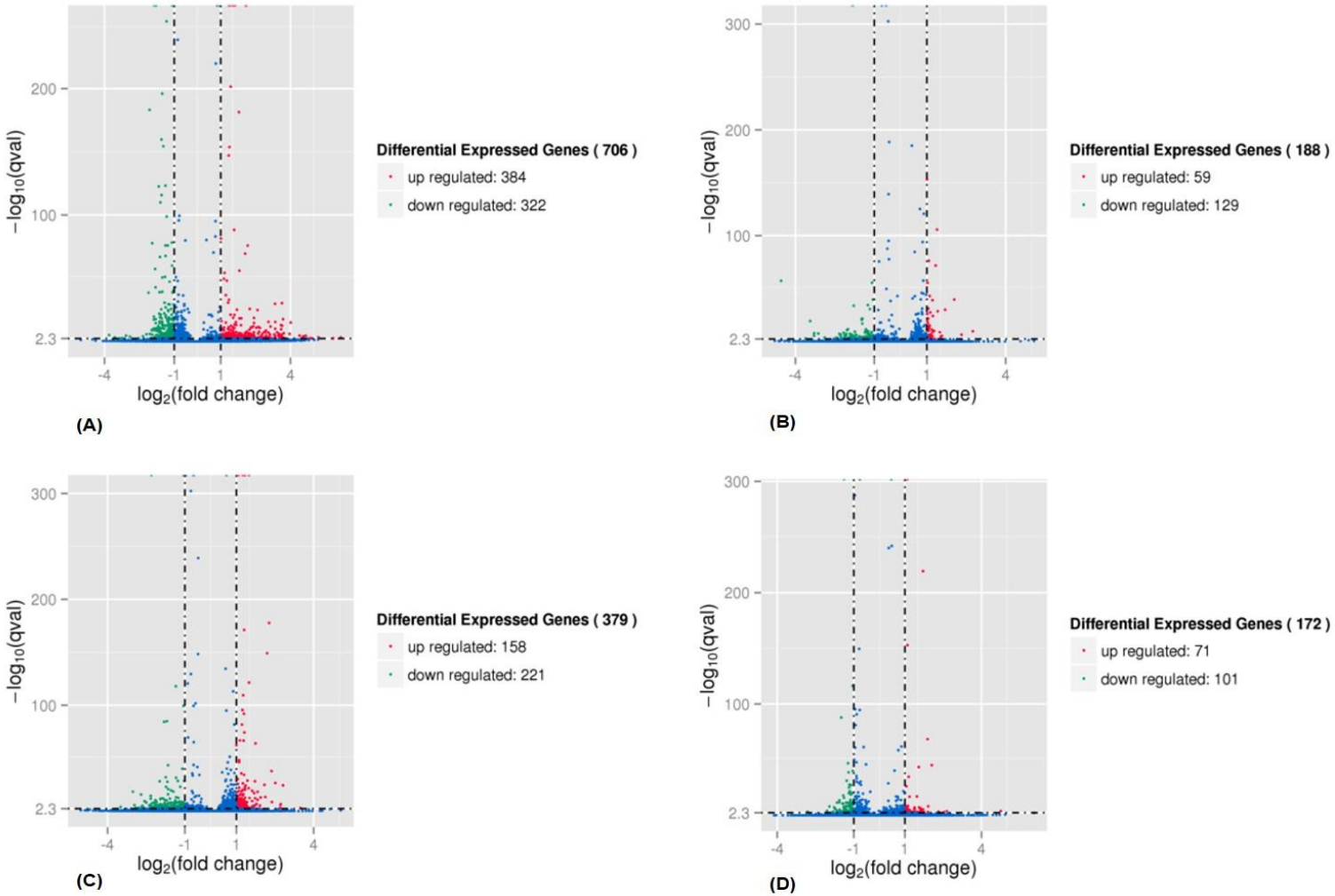

Figure 1. Volcano plots inferring the overall distribution of differentially expressed genes (q<0.05) in maize following infection with *Fusarium verticillioides* at (A) 24 hours after inoculation (hai), (B) 48 hai, (C) 72 hai and (D) 7 days after inoculation.

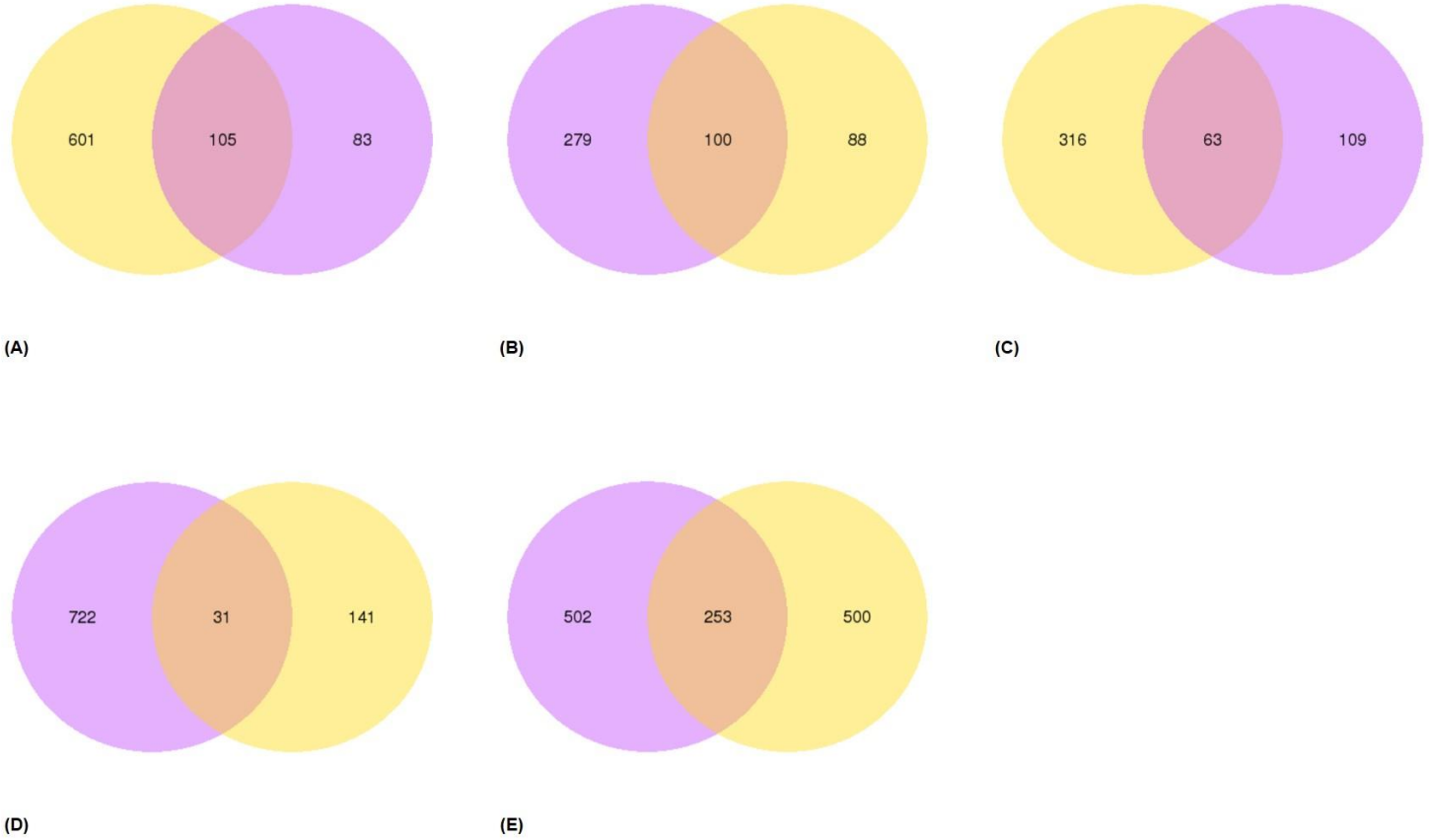

Figure 2. Venn diagrams representing the number of differentially expressed genes following infection of a maize inbred line with *Fusarium verticillioides* at (A) 48 hours after inoculation (hai) vs. 24 hai, (B) 72 vs. 48 hai and (C) 7 days vs. 72 hai.

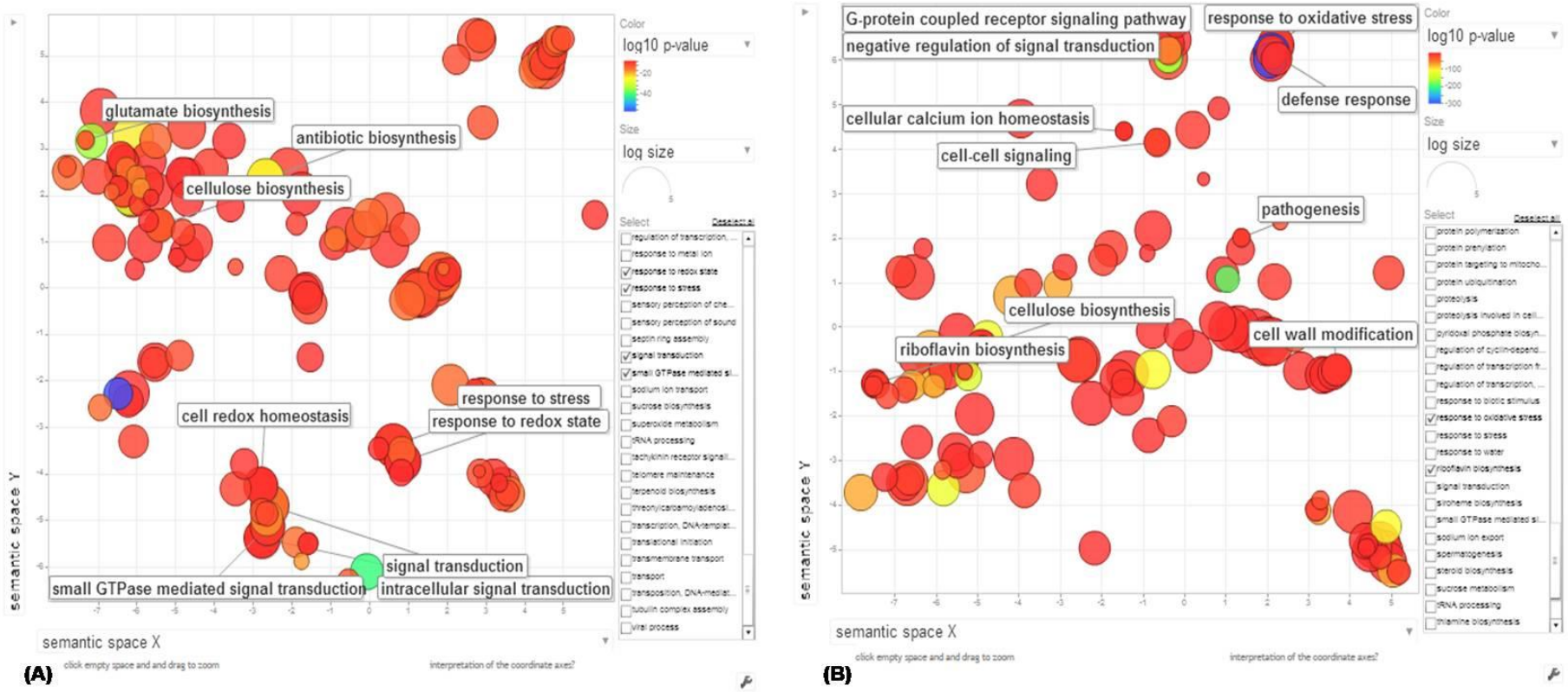

Figure 3. Semantic similarity-based Gene Ontology (GO) scatterplots of the differentially expressed genes (A) up-regulated and (B) down-regulated at 24 hours after inoculation.

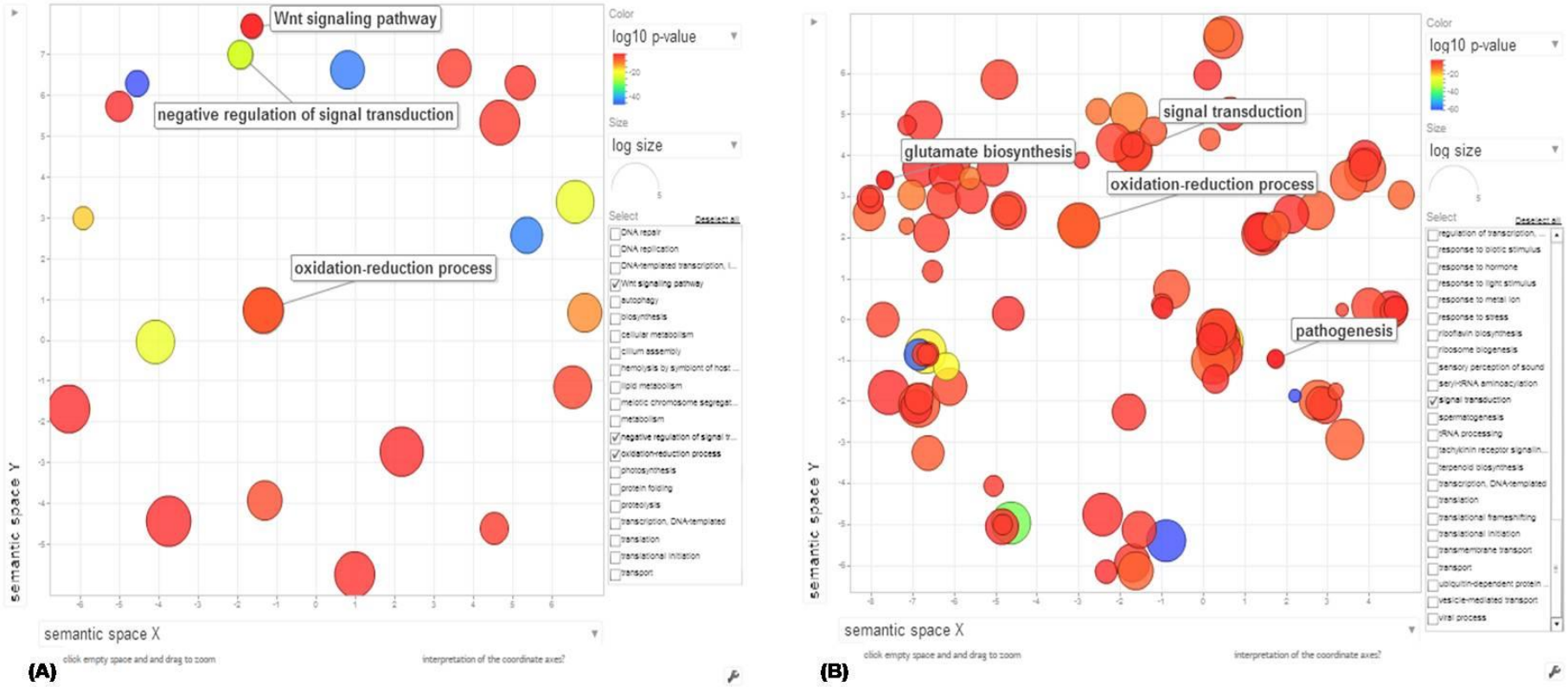

Figure 4. Semantic similarity-based Gene Ontology (GO) scatterplots of the differentially expressed genes (A) up-regulated and (B) down-regulated at 48 hours after inoculation.

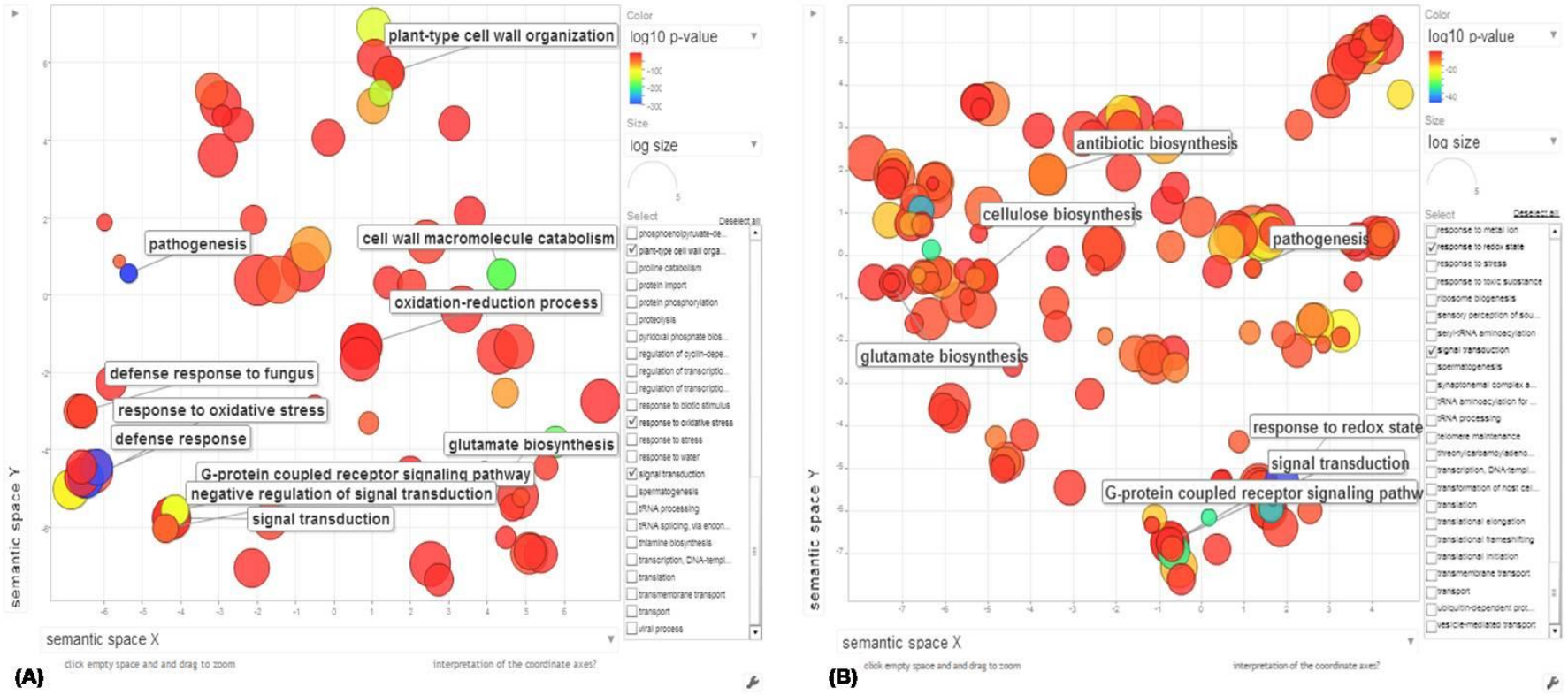

Figure 5. Semantic similarity-based Gene Ontology (GO) scatterplots of the differentially expressed genes (A) up-regulated and (B) down-regulated at 72 hours after inoculation.

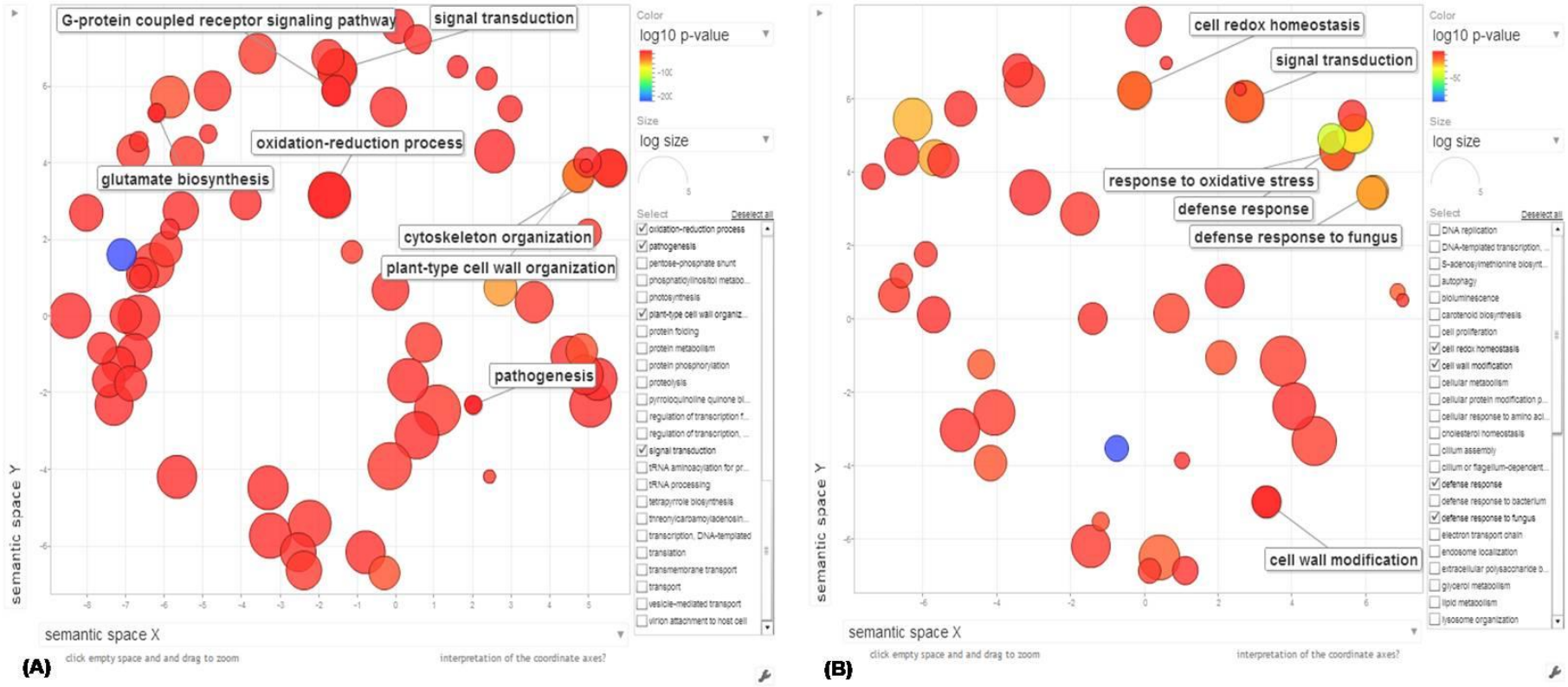

Figure 6. Semantic similarity-based Gene Ontology (GO) scatterplots of the differentially expressed genes (A) up-regulated and (B) down-regulated at 7 days after inoculation.

CHAPTER 6

Conclusion

Maize is an important food and feed crop produced worldwide, and is one of the most important staple foods produced in Africa (Byerlee and Eicher, 1997). The production of maize in Africa, however, is met with numerous constraints, of which ear rot diseases are of significant importance due to food security and food safety issues (Wagacha and Muthomi, 2008). Fusarium ear rot (FER), caused by *Fusarium verticillioides* and Aspergillus ear rot (AER), caused by *Aspergillus flavus*, are considered the most important ear rot diseases in southern and eastern Africa, respectively. In addition, *F. verticillioides* contaminates maize grain with mycotoxins called fumonisins, and *A. flavus* contaminates grain with aflatoxins. The ubiquitous contamination of African-produced maize with mycotoxins can lead to daily intake levels that result in acute and prolonged negative effects on human health. Of the management strategies available to reduce ear rot diseases and mycotoxin contamination of maize grain, host-plant resistance provides the most feasible, environmentally sound and cost-effective means of reducing contamination with mycotoxigenic fungi in the field (Afolabi *et al*., 2007).

The response of South Africa maize inbred lines to *F. verticillioides* infection and fumonisin accumulation was determined in multiple environments. All maize cultivars produced commercially in the country have insufficient levels of resistance to *F. verticillioides* (Rheeder *et al*., 1990; Janse van Rensburg *et al*., 2015), and new FER- and fumonisin-resistant cultivars are thus required. Four inbred lines (CML 390; RO 424W, US 2540W and VO 617y-2) were consistently resistant to the fungus and its toxins across localities over two seasons. They also demonstrated good stability in their response to *F. verticillioides*, confirming their broad-adaptability. The resistant lines can therefore be used as a source of resistance in breeding programmes for the development of new locally-adapted resistant maize hybrids. They also can be of value in research efforts to identify quantitative trait loci for marker-assisted selection, and for discovering genes responsible for resistance for genetic modification of maize.

Kenyan maize inbred lines resistant to *A. flavus* and aflatoxins were evaluated for resistance to *F. verticillioides* and fumonisin accumulation in both South Africa and Kenya. This was the first time the Kenyan material has been screened for resistance to *F. verticillioides* and fumonisins. Several of the Kenyan maize lines outperformed the most resistant South African in both countries. Kenyan inbred line CML 495 was consistently the most resistant to FER and fumonisin contamination, but not significantly more than Kenyan lines CML 264 and CKL05015 and the South African line RO 549W. This study demonstrated that AER/aflatoxin-resistant lines may serve as a source of resistance to *F. verticillioides* and fumonisin accumulation. It also showed that fumonisin contamination of maize grain in Kenya can be severe, as Kiboko in Kenya was found to be the ideal site for FER and fumonisin production in maize grain.

Gamma irradiation is a technology that has become popular for mutation breeding of agricultural crops to generate diversity for increased yields, better agronomic features, and disease resistance. It generates mutants that are stable in a more efficient and publically acceptable way than conventional breeding and genetic modification, respectively. In this study gamma irradiation was used for the first time to mutate elite inbred lines for increased resistance to *F. verticillioides* and fumonisins. M_4 selections derived from the inbred lines proved to be more resistant to FER, *F. verticillioides* colonisation and fumonisin accumulation than their non-irradiated parents. This result provides a basis to study genomic alterations that contribute to enhanced resistance to *F. verticillioides* and fumonisin accumulation by means of advanced biotechnological tools such as next-generation sequencing. The mutants generated in this study still need to be further screened for good agronomic traits (yield, plant height, growth cycle) and general combinability.

The transcriptional response of maize to infection by *F. verticillioides* was studied over a period of 7 days to identify defence-related plant responses and identify genes that could potentially be used for enhanced plant resistance. Transcripts associated with pathogen recognition, signalling molecules, pathogenesis-related genes, cell wall restructuring and secondary hormone-based signalling were induced while *F. verticillioides* colonisation of maize grain continued to increase. The rate of gene expression in maize plants indicated a delayed response based on a pathogen-associated molecular pattern immunity response. Gene expression in resistant and susceptible maize genotypes should be compared in future, and the importance of genes in the defence response confirmed by gene knock-out studies.

This study has provided new information on resistance in maize to *F. verticillioides* and fumonisins. South African maize inbred lines and AER/aflatoxin-resistant maize lines from Kenya have been identified as potential sources of resistance for the development of resistant maize hybrids. The ability of gamma radiation to induce genetic variation for resistance in elite inbred lines has also been demonstrated. Inbreeding of the maize mutants should continue to the M_6-M_8 generation to ensure homozygosity, and further evaluated in multiple environments to determine broad or specific adaptability for disease resistance. The evaluation of the transcriptional response in maize to *F. verticillioides* 7 days after infection has identified differentially expressed genes that could potentially be used as molecular markers or for genetic engineering to develop maize hybrids resistant to FER and fumonisins.

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